	L #	Hits	Search Text	DBs	Time Stamp
1	L1	241	sulfolobus or acidocaldarius	USPAT	2000/10/19 13:50
2	L2	2860	trehalose	USPAT	2000/10/19 13:50
3	L3	23	1 and 2	USPAT	2000/10/19 14:14

	Document ID	Issue Date	Pages	Title
1	US 6129788 A	20001010	19	Method of producing saccharide preparations
2	US 6100073 A	20000808	19	Acid-stable and thermo-stable enzymes derived from sulfolobus species
3	US 6087149 A	20000711	27	Starch conversion process
4	US 6066492 A	20000523	12	Microorganism capable of degrading polylactic acid resin and method of degrading polylactic acid resin using said microorganism
5	US 6046018 A	20000404	18	Highly sensitive method for assaying chiro-inositol and compositions for the assay
6	US 6027918 A	20000222	28	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
7	US 6017899 A	20000125	30	Non-reducing saccharide-forming enzyme, its preparation and uses
8	US 5976856 A	19991102	27	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
9	US 5925556 A	19990720	12	Method of degrading polylactic acid resin using staphylococcus hominis and staphylococcus epidermidis
10	US 5922578 A	19990713	28	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide

	Document ID	Issue Date	Pages	Title
11	US 5919668 A	19990706	40	Non-reducing saccharide and its production and use
12	US 5863771 A	19990126	13	Saccharide composition comprising maltooligosylturanose and maltooligosylpalatinose, its preparation and uses
13	US 5861295 A	19990119	12	Nucleic acid-free thermostable enzymes and methods of production thereof
14	US 5856146 A	19990105	27	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
15	US 5789392 A	19980804	40	Saccharide composition with reduced reducibility, and preparation and uses thereof
16	US 5723327 A	19980303	22	Thermostable trehalose-releasing enzyme, and its preparation and uses
17	US 5716838 A	19980210	30	Non-reducing saccharide-forming enzyme, its preparation and uses
18	US 5714368 A	19980203	19	Thermostable non-reducing saccharide-forming enzyme its production and uses
19	US 5681826 A	19971028	41	Saccharide composition with reduced reducibility, and preparation and uses thereof
20	US 5677442 A	19971014	30	Method of crystallizing trehalose without using organic solvent
21	US 5658765 A	19970819	14	Xylanase process for producing the same method for the treatment of pulp and production of xylo-oligosaccharides
22	US 5610047 A	19970311	29	Non-reducing saccharide-forming enzyme, its preparation and uses

	Document ID	Issue Date	Pages	Title
23	US 5356790 A	19941018	18	Highly sensitive assay method for myo-inositol, composition for practicing same, novel myo-inositol dehydrogenase, and process for producing same

USPT

US-CL-CURRENT: 435/105,435/96 ,435/98

US-PAT-NO: 6129788
DOCUMENT-IDENTIFIER: US 6129788 A
TITLE: Method of producing saccharide preparations
DATE-ISSUED: October 10, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Liaw; Gin C.	Decatur	IL	N/A	N/A
Pedersen; Sven	Gentofte	N/A	N/A	DKX
Hendriksen; Hanne Vang	Holte	N/A	N/A	DKX
Svendsen; Allan	Birker.o	N/A	N/A	DKX
Nielsen; Bjarne R.o	slashed.d	N/A	N/A	DKX
slashed.nfeldt	Virum	N/A	N/A	DKX
Nielsen; Ruby Illum	Farum			

US-CL-CURRENT: 127/40,435/105 ,435/96 ,435/98
ABSTRACT:
The present invention relates to a method for the production of saccharide preparations, i.e., syrups, by saccharifying a liquefied starch solution, which method comprises a saccharification step during which step one or more enzymatic saccharification stages takes place, and the subsequent steps of one or more high temperature membrane separation steps, and recirculation of the saccharification enzyme, in which method the membrane separation steps are carried out as an integral part of the saccharification step.
16 Claims, 5 Drawing figures
Exemplary Claim Number: 1,9
Number of Drawing Sheets: 5

BSPR:
The present invention relates to the production of mono and/or oligosaccharides from starch, including dextrose, trehalose, isomaltooligosaccaharides, cyclodextrins and maltooligosaccharides. In a specific aspect, the invention provides a method of saccharifying a liquefied starch solution, which method comprises a saccharification step during which step one or more enzymatic saccharification stages takes place, and the subsequent steps of one or more high temperature membrane separation steps, and re-circulation of the saccharification enzyme, in which method the membrane separation steps are carried out as an integral part of the saccharification step.

BSPR:
In another specific aspect, the invention provides a method of producing a mono and/or oligosaccharide, such as dextrose, trehalose, isomaltooligosaccaharide, cyclodextrins and maltooligosaccharide preparation, which method comprises an enzymatic saccharification step, and the subsequent steps of one or more high temperature membrane separation steps and re-circulation of the saccharification enzyme.

BSPR:
Trehalose (.alpha.-D-glucopyranosyl .alpha.-D-glucopyranoside) is a non reducing disaccharide with two glucose residues bound by a .alpha.-1,1 linkage.

BSPR:
Enzymatic processes for producing trehalose from starch or maltooligosaccharides are described by, e.g., Kato et al., (1996), Biosci. Biotech. Biochem., 60 (3), p. 546-550); Kazuhisa et al. (1997), Starch 49, no. 1. p. 26-30; and in EP 764,720.

BSPR:
It has now been found that in a method of producing mono and/or oligosaccharides from starch, including dextrose, trehalose, isomaltooligosaccharides, cyclodextrins and maltooligosaccharides, the efficiency can be improved significantly, and the costs lowered, if in the saccharification (or hydrolyzing) step, after the liquefaction step, the syrup is subjected to one or more high temperature membrane separation steps, and the

saccharification enzyme is returned to the saccharification step. According to the method of the present invention, the membrane separation step may be regarded as an integral part of the saccharification step.

BSPR:
When producing saccharides with more than one saccharide unit, i.e., **trehalose,** isomaltooligosaccharides, cyclodextrins and maltooligosaccharides the hydrolyzing step (after the liquefaction step) is followed by an ultra and microfiltration step or a micro and ultrafiltration step.

BSPR:
In its second aspect, the invention provides a method for the production of a mono and/or oligosaccharide preparation of, e.g., dextrose, **trehalose,** isomaltooligosaccharides, cyclodextrins and maltooligosaccharides, which method comprises an enzymatic saccharification step, and the subsequent steps of

BSPC:
Trehalose Syrups

DEPR:
According to the method of the invention, the retentate from the membrane separation is conveyed back (re-circulated) to the saccharification step. Preferably the retentate from the membrane separation is re-circulated to a saccharification stage in the saccharification step, at which stage the content of the reaction mixture matches the content of the retentate with respect to the saccharide, such as glucose, **trehalose,** isomaltooligosaccharide, cyclodextrin or maltooligosaccharide.

DEPR:
When producing **trehalose,** the feed stream subjected to membrane separation originating from the saccharification stage holds of from about 50 to about 90%, preferably of from about 60 to 90%, more preferred of from about 75-90% **trehalose**.

DEPR:
In the context of the present invention a membrane separation step comprises a microfiltration step followed by an ultrafiltration step or an ultrafiltration step followed by a microfiltration step when producing **trehalose,** isomaltooligosaccharides, cyclodextrins and maltooligosaccharides.

DEPR:
In a preferred embodiment, the membrane separation steps comprises a microfiltration step and an ultrafiltration step, applied in the order specified. This embodiment is particularly useful for the production of a syrup holding from about 95 to about 96% glucose, or from 10-40% isomaltose, or 30 to above 80% maltose, or 75-90% **trehalose,** or 30-60% cyclodextrins.

DEPR:
In the saccharification step, when producing **trehalose,** liquefied starch is subjected to the action of an enzyme capable of first converting maltooligosaccharide (from the liquefaction step) into the non reducing saccharide maltooligosyl **trehalose** by intramolecular transglycosylation followed by a subsequent step of hydrolyzing the reaction product of the first step (i.e., maltooligosyl **trehalose) into trehalose**. The saccharification step may be performed using maltooligosyl **trehalose** synthase (MTSase) and maltooligosyl **trehalose** trehalohydrolase (MTHase), e.g., the two enzymes described by Masaru et al. (1996), Biosci. Biotech. Biochem., 60 (3), 546-550). MTSase and MTHase act on amylose or starch to produce **trehalose**.

DEPR:
Another enzymatic process for producing **trehalose** from starch or maltooligosaccharides (see Kato et al., (1996), Biosci. Biotech. Biochem., 60 (3), p. 546-550) involves using **trehalose**-producing enzymes, a glycosyltransferase and an amylase, respectively, from the hyperthermophilic archae **Sulfolobus** solfataricus KM1.

DEPR:
Further, EP 764720 also describes using two enzymes from Solfolobus spp. for

preparing **trehalose** from starch or maltooligosacchairdes.

DEPR:
When producing **trehalose,** the feed stream subjected to membrane separation originating from the saccharification stage holds of from about 50 to about 90%, preferably of from about 60 to 90%, more preferred of from about 75-90% **trehalose**.

DEPR:
A thermostable isoamylase may be derived from a strain of Flavobacterium, in particular Flavobacterium odoratum, a strain derived from the thermophilic acrhaebacterium **Sulfolobus acidocaldarius** (Hayashibara, (1996) Biochimica et Biophysica Acta 1291, p. 177-181, such as **Sulfolobus acidocaldarius** ATCC33909 and from a strain of Rhodethermus marius.

DEPR:
Preferably, the saccharification step, when producing **trehalose** is performed in presence of a MTSase and MTHase, e.g., the enzymes disclosed by Masaru et al. (1996), Biosci. Biotech. Biochem., 60 (3), 546-550).

DEPL:
Production of **Trehalose** (containing 75-90% **trehalose**)

USPT

US-CL-CURRENT: 435/105,435/202 ,435/203 ,435/204 ,435/96

US-PAT-NO: 6100073
DOCUMENT-IDENTIFIER: US 6100073 A
TITLE: Acid-stable and thermo-stable enzymes derived from **sulfolobus** species
DATE-ISSUED: August 8, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Deweer; Philippe	Aalst	N/A	N/A	BEX
Amory; Antione	Rixensart	N/A	N/A	BEX

US-CL-CURRENT: 435/99,435/105 ,435/202 ,435/203 ,435/204 ,435/96
ABSTRACT:
Novel acid-stable and thermo-stable enzymes having .alpha.-1,4 hydrolytic activity and a .alpha.-1,6 hydrolytic activity which are derived from strains of the genus **Sulfolobus**. These enzymes are capable of expressing high levels of .alpha.-1,4 hydrolytic activity, including the maximum .alpha.-1,4 hydrolytic activity thereof, at highly acidic pHs of between about 2.5 and about 4.5. These .alpha.-amylases are further capable of expressing high levels of .alpha.-1,4 hydrolytic activity, including the maximum .alpha.-1,4 hydrolytic activity thereof, at high temperatures of between about 90.degree. C. and about 120.degree. C. Particularly disclosed herein are such enzymes which are derived from strains of the species S. **acidocaldarius** and, in particular, **Sulfolobus acidocaldarius** DSM 639. Modified starch degradation (liquefaction and saccharification) processes using these novel enzymes are also disclosed herein.
33 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

TTL:
Acid-stable and thermo-stable enzymes derived from **sulfolobus** species

ABPR:
Novel acid-stable and thermo-stable enzymes having .alpha.-1,4 hydrolytic activity and a .alpha.-1,6 hydrolytic activity which are derived from strains of the genus **Sulfolobus**. These enzymes are capable of expressing high levels of .alpha.-1,4 hydrolytic activity, including the maximum .alpha.-1,4 hydrolytic activity thereof, at highly acidic pHs of between about 2.5 and about 4.5. These .alpha.-amylases are further capable of expressing high levels of .alpha.-1,4 hydrolytic activity, including the maximum .alpha.-1,4 hydrolytic activity thereof, at high temperatures of between about 90.degree. C. and about 120.degree. C. Particularly disclosed herein are such enzymes which are derived from strains of the species S. **acidocaldarius** and, in particular, **Sulfolobus acidocaldarius** DSM 639. Modified starch degradation (liquefaction and saccharification) processes using these novel enzymes are also disclosed herein.

BSPR:
The present invention relates to novel acid-stable and thermo-stable enzymes having an .alpha.-1,4 hydrolytic activity and which are derived from strains of the genus **Sulfolobus**, and in particular strains of the species **Sulfolobus acidocaldarius**, and the use of these novel enzymes in starch degradation.

BSPR:
Despite the advantages that such an enzyme, would provide, especially in the production of sugars from starch, to the best of our knowledge, no such .alpha.-amylase has been previously identified, isolated and/or purified. This is despite the fact that strains of the genus **Sulfolobus** and, in particular, the strain from which the enzyme of the present invention is derived, **Sulfolobus acidocaldarius** DSM 639, have been known, deposited in an-approved culture collection and available to the general public for a number of years.

BSPR:

While .alpha.-amylases derived from other strains of Sulfolobus are known, these .alpha.-amylases are not known to possess the properties (especially the desired acid-stable properties) described above. An example of such other known .alpha.-amylases derived from other species of Sulfolobus include that .alpha.-amylase which is derived from the species Sulfolobus solfataricus (see Lama et al., Biotechnology Letters, 1990, 12: 431-432 and Lama et al., Biotech Forum Europe, 1991, 8:201-203). That .alpha.-amylase (which is very different from the .alpha.-amylase of the present invention) possesses an optimum pH of 5.5 and an optimum temperature of 70.degree. C. Unfortunately, both of these properties would restrict the use of the .alpha.-amylases from such species of Sulfolobus, as they also restrict the use of the conventional .alpha.-amylases, in a number of industrial applications, including liquefaction in the enzymatic degradation starch to sugars.

BSPR:
Further problems presented by the use of the .alpha.-amylase derived from S. solfataricus in liquefaction is that it is produced intracellularly and that it catalyzes the synthesis of **trehalose**. In fact, we are not aware of any acid-stable .alpha.-amylases which are extracellularly-secreted by any species of Sulfolobus.

BSPR:
Amylopullulanases are lesser-known hydrolyzing enzymes having .alpha.-1,4 hydrolyzing activity in amylose and starch as well as .alpha.-1,6 hydrolyzing activity in pullulan and starch. Amylopullulanases are known to be naturally-produced by species of the genra Bacillus, Thermus, Clostridium, Thermoanaerobium, Thermoanaerobacter, Pyrococcus and Thermococcus. However, we are not aware of any such amylopullulanase which is derived from any species or strain of the genus Sulfolobus.

BSPR:
In accordance with the teachings of the present invention, disclosed herein are novel enzymes having .alpha.-1,4 hydrolytic activity in starch which are derived from species of the genus Sulfolobus including, in particular, the species Sulfolobus acidocaldarius. Specifically disclosed herein, as an example of such enzymes, is the enzyme having such .alpha.-1,4 hydrolytic activity which is derived from the strain Sulfolobus acidocaldarius DSM 639.

BSPR:
As used herein in referring to enzymes, nucleotides and microbial (i.e., Sulfolobus) strains, the term "derived from" means that the enzymes and nucleotides being spoken of are native to (originate from) the particular microbial strain from which they are said to be "derived from". In this regard, enzymes and nucleotides derived from S. acidocaldarius DSM 639 refer to those enzymes and nucleotides which are native to (originate from) S. acidocaldarius DSM 639. This defintion includes enzymes and nucleotide sequences which are identical to those enzymes and nucleotide sequences spoken of but which have (in the case of nucleotides) been either inserted into (used to transform) a suitable host organism, and (in the case of the enzyme) which has been secreted by a transformed host. This defintion also includes mutants, variants and derivatives of the enzymes and nucleotides referred to.

BSPR:
As used herein, the term "mutants and variants", when referring to microbial strains (such as S. acidocaldarius DSM 639), refers to cells obtained by alteration of the DNA nucleotide sequence of, for example, the structural gene coding for the enzyme thereof having the .alpha.-1,4 hydrolytic activity.

BSPR:
In still yet further accordance with the teachings of the present invention, there is disclosed herein an enzyme having .alpha.-1,4 hydrolytic activity in starch which are derived from a species of Sulfolobus, and which enzymes have an estimated molecular weight of about 95 kDa and/or an optimum pH of between about 3.0 and about 4.0 (about 3.5) and/or an optimum temperature of between about 110.degree. C. and about 115.degree. C.

BSPR:
In yet further accordance with the teachings of the present invention,

disclosed herein are novel enzymes having .alpha.-1,4 hydrolytic activity in starch which enzyme is derived from species of the genus Sulfolobus, such as strains of the species S. acidocaldarius, (including, in particular Sulfolobus acidocaldarius DSM 639), which enzymes are capable of expressing the maximum .alpha.-1,4 hydrolytic (enzymatic) activity thereof in the pH range that can be encountered in liquefaction, such that the necessity of adjusting the pH of, and/or of increasing the calcium ion concentration of, the starch slurry during liquefaction is avoided. Preferably, this enzyme further has an .alpha.-1,6 hydrolytic activity in starch.

BSPR:
In still yet further accordance with the teachings of the present invention, disclosed herein are novel enzymes having .alpha.-1,4 hydrolytic activity in starch which are derived from species of the genus Sulfolobus, such as strains of the species Sulfolobus acidocaldarius (including, in particular, Sulfolobus acidocaldarius DSM 639), and which enzymes are capable of expressing the maximum .alpha.-1,4 hydrolytic (enzymatic) activity in the temperature range (between about 90.degree. C. and about 110.degree. C.) that can be encountered in liquefaction. Preferably, this enzyme further has an .alpha.-1,6 hydrolytic activity in starch.

BSPR:
In yet still further accordance with the teachings of the present invention, disclosed herein are novel enzymes having an .alpha.-1,4 hydrolytic activity in starch which are derived from species of the genus Sulfolobus, such as strains of the species Sulfolobus acidocaldarius (including, in particular, Sulfolobus acidocaldarius DSM 639), which enzymes are capable of expressing the maximum .alpha.-1,4 hydrolytic (enzymatic) activity thereof in both the pH ranges and the temperature ranges that can be encountered in the liquefaction step, such that the necessity of either adjusting the pH of, and/or of increasing the calcium ion concentration of, the starch slurry during liquefaction is avoided.

DEPR:
The hydrolyzing enzymes of the present invention are novel enzymes having .alpha.-1,4 hydrolytic activity in starch and .alpha.-1,6 hydrolytic activity in starch which are derived from strains (and natural isolates) of the genus Sulfolobus, such as strains of the species S. acidocaldarius, S. brierleyi (Acidianus brierleyi), S. metallicus, S. shibatae and S. solfataricus.

DEPR:
The enzymes of the present invention include that enzyme having .alpha.-1,4 hydrolytic activity in starch which is derived from the strain Sulfolobus acidocaldarius DSM 639.

DEPR:
The strain Sulfolobus acidocaldarius has been deposited in the Deutsche Sammlung von Mikroorganismen (DSM) located at Mascheroder Weg 1b Braunschweig, Federal Republic of Germany, under accession number DSM 639. This strain is publically-accessible.

DEPR:
These enzymes are extracellularly secreted by the strains of the genus Sulfolobus (such as the strain S. acidocaldarius DSM 639 and other strains of S. acidocaldarius, S. brierleyi, S. metallicus, S. shibatae and S. solfataricus) into the fermentation broth.

DEPR:
For purposes of illustration of the enzymes of the present invention, the properties thereof will be hereafter discussed by reference to those properties and characteristics of that enzyme derived from S. acidocaldarius DSM 639, which is believed to be representative of these acid and thermal stable enzymes having .alpha.-1,4 hydrolytic activity in starch which are disclosed herein that are naturally (extracellularly) secreted by other strains of the genus Sulfolobus, including other strains of the species S. acidocaldarius.

DEPR:
The enzymes of the present invention may be homologously expressed and extraceliularly secreted into the culture broth by strains of the genus

Sulfolobus, such as S. **acidocaldarius** DSM 639, in their normal, native fashion.

DEPR:
Such recombinant expression may be homologous by another culture of the same strain of **Sulfolobus** (such as S. **acidocaldarius** DSM 639). Alternatively, such recombinant expression may be heterologous by another strain of the same species of **Sulfolobus** (such as another strain of S. **acidocaldarius**) and/or a strain a different species of the genus **Sulfolobus** (such as another strain of the species S. Brierleyi, S. metallicus, S. shibatae and S. solfataricus) and/or a strain of another genus entirely, such as strains of Bacillus (for example, strains of Bacillus licheniformis, B. subtilis, B. alkalophilus, B. lentus, B. pumilus and B. Amyloliquefaciens) and fungal strains, such as strains of Aspergillus (such as Aspergillus niger) and Rhizopus to name but two.

DEPR:
The novel enzymes of the present invention may be produced by cultivation of strains of **Sulfolobus** (such as S. **acidocaldarius** DSM 639) under aerobic conditions in nutrient medium containing assimilable carbon and nitrogen together with other essential nutrient(s). The medium can be composed in accordance with principles well-known in the art.

DEPR:
A freeze-dried culture of **Sulfolobus acidocaldarius** DSM 639 was obtained from the Deutsche Sanmlung von Mikroorganismen (Germany), which had been deposited therein under accession number DSM 639.

DEPR:
Five (5) sterile 100 ml screw-cap bottles, each containing 40 ml of the culture medium were then inoculated with respective 200 .mu.l quantities of the S. **acidocaldarius** DSM 639 culture suspension.

DEPR:
culture sample of S. **acidocaldarius** DSM 639.

DEPR:
The fermentor was then inoculated with the two liter culture of S. **acidocaldarius** DSM 639, obtained as described above.

DEPR:
According to the results of this assay, the optimum pH of the enzyme which is derived from **Sulfolobus acidocaldarius** DSM 639 (for the expression of the .alpha.-1,4 hydrolytic activity thereof) is about pH 3.5

DEPR:
The optimum temperature of the enzyme derived from **Sulfolobus acidocaldarius** DSM 639 was determined by running the standard assay described above in Example 4 on each of the tubes (samples) at a constant pH of 3.5, and at various temperatures, ranging from 60 to 120.degree. C. (.+-.2.degree. C.). The results of such assays were then analyzed in the manner that was also described above in Example 4 for the standard assay.

DEPR:
According to this table, the optimum temperature of the enzyme derived from **Sulfolobus acidocaldarius** DSM 639 (for the expression of the .alpha.-1,4 hydrolytic activity thereof) is about 115.degree. C.

DEPR:
The influence of various metal cations and of EDTA on the .alpha.-1,4 hydrolytic activity of the enzyme derived from **Sulfolobus acidocaldarius** DSM 639 was determined by running the standard assay described above in Example 4 on each of the tubes (samples) in the presence of the additional various metal cations at a final concentration of 2, 5, and 10 mM. The results of such assays were then analyzed in the manner that was also described above in Example 4 for the standard assay.

DEPC:
Enzyme Production by **Sulfolobus acidocaldarius** DSM 639

CLPR:
1. An isolated enzyme comprising .alpha.-1,4 hydrolytic activity, characterized in that the enzyme is derived from a strain of the genus **Sulfolobus** and is capable, in pHs of 4.5 and below, of expressing the maximal .alpha.-1,4 hydrolytic activity thereof.

CLPR:
27. The isolated enzyme according to claim 1, wherein the enzyme is derived from a strain of the species **Sulfolobus acidocaldarius**.

CLPR:
28. The isolated enzyme according to claim 27, wherein the enzyme is derived from the strain **Sulfolobus acidocaldarius** DSM 639.

CLPR:
30. An enzymatic composition comprising an enzyme obtained from a strain of the genus **Sulfolobus** having .alpha.-1,4 hydrolytic activity wherein said enzyme is capable, in pHs of 4.5 and below, of expressing the maximal .alpha.-1,4 hydrolytic activity thereof.

CLPR:
31. The enzymatic composition according to claim 30, wherein the enzyme is derived from a strain of the species **Sulfolobus acidocaldarius**.

CLPR:
32. The enzymatic composition according to claim 31, wherein the enzyme is derived from the strain **Sulfolobus acidocaldarius** DSM 639.

CLPR:
33. An isolated enzyme comprising .alpha.-1,4 hydrolytic activity, wherein the enzyme is derived from a strain of the genus **Sulfolobus**, has an estimated molecular weight of about 95 kDa, and has an optimum pH of about 3.5 and an optimum temperature of 110-115.degree. C. for the expression of .alpha.-1,4 hydrolytic activity thereof.

ORPL:
Lama, et al, "Starch Conversion With Immobilized Thermophilic Archaebacterium **Sulfolobus** Solfataricus ," Biotechnology Letters, vol. 12: pp.431-432 (1990).

USPT

US-CL-CURRENT: 435/252.3,435/252.31 ,435/252.33 ,435/254.11 ,435/254.2 ,435/254.21 ,435/254.22 ,435/254.23 ,435/254.3 ,435/254.6 ,435/254.7 ,435/320.1 ,435/325 ,536/23.1 ,536/23.2

US-PAT-NO: 6087149
DOCUMENT-IDENTIFIER: US 6087149 A
TITLE: Starch conversion process
DATE-ISSUED: July 11, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tsutsumi; Noriko	Chiba-ken	N/A	N/A	JPX
Bisg.ang.rd-Frantzen; Henrik	Bagsv.ae	N/A	N/A	DKX
Svendsen; Allan	butted.rd Birker.o slashed.d	N/A	N/A	DKX

US-CL-CURRENT: 435/210,435/252.3 ,435/252.31 ,435/252.33 ,435/254.11 ,435/254.2 ,435/254.21 ,435/254.22 ,435/254.23 ,435/254.3 ,435/254.6 ,435/254.7 ,435/320.1 ,435/325 ,536/23.1 ,536/23.2

ABSTRACT:
The present invention relates to a starch conversion process of the type which includes a debranching step wherein an isoamylase being active at the process conditions prevailing is used for debranching the starch and to the use of thermostable isoamylases for starch conversion. The invention further relates to an isolated isoamylase obtained from a strain of the genus Rhodothermus and to cloned DNA sequences encoding isoamylases derived from a strain of Rhodothermus or **Sulfolobus,** to expression vectors comprising said DNA sequence, host cells comprising such expression vectors, and finally to methods for producing said isoamylases.
23 Claims, 7 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

ABPL:
The present invention relates to a starch conversion process of the type which includes a debranching step wherein an isoamylase being active at the process conditions prevailing is used for debranching the starch and to the use of thermostable isoamylases for starch conversion. The invention further relates to an isolated isoamylase obtained from a strain of the genus Rhodothermus and to cloned DNA sequences encoding isoamylases derived from a strain of Rhodothermus or **Sulfolobus,** to expression vectors comprising said DNA sequence, host cells comprising such expression vectors, and finally to methods for producing said isoamylases.

BSPR:
The invention relates to a starch conversion process of the type which includes a debranching step. The invention also relates to the use of a thermostable isoamylase for debranching starch. The invention further relates to an isolated isoamylase obtained from a strain of the genus Rhodothermus, to cloned DNA sequences encoding isoamylases derived from a strain of Rhodothermus or **Sulfolobus,** to expression vectors comprising said DNA sequence, host cells comprising such expression vectors, and finally to methods for producing said isoamylases.

BSPR:
Starches such as corn, potato, wheat, manioc and rice starch are used as the starting material in commercial large scale production of sugars, such as high fructose syrup, high maltose syrup, maltodextrins amylose, **trehalose,** G2-G8 oligosaccharides (including functional oligosaccharides) and other carbohydrate products such as fat replacers.

DRPR:
FIG. 5 shows the pH curve of **Sulfolobus acidocaldarius** isoamylase.

DEPR:

Specific examples of thermostable debranching enzymes are the thermostable isoamylases derived from the thermophilic bacteria such as Sulfolobus acidocaldarius ATCC 33909 (Maruta, K. et al., Biochimica et Biophysica Acta 1291, p. 177-181 (1996)), Sulfolobus solfataricus ATCC 35092 (accession number: Y08256) and Rhodothermus marinus DSM 4252 as will be described further below.

DEPR:
Isoamylases which can be used according to the invention include the thermostable isoamylase derived from the thermophilic archaebacteria Sulfolobus acidocaldarius, Sulfolobus solfataricus and the thermophilic eubacterium Rhodothermus marinus (as will be described in details below).

DEPR:
In the case of the starch conversion process is a starch depolymerization process the thermostable isoamylase is used in combination with an .alpha.-amylase during the liquefaction step. The thermostable isoamylase may be derived from the thermophilic archaebacteria Sulfolobus acidocaldarius or Sulfolobus solfataricus or from Rhodothermus marinus.

DEPR:
The homology search showed that the most related known sequence(s) were isoamylases from Sulfolobus acidocaldarius and Sulfolobus solfataricus. The DNA sequence of the invention (SEQ ID NO: 3) encoding an isoamylase shows about 51-52% DNA homology to the known isoamylase sequences from Sulfolobus acidocaldarius and Sulfolobus solfataricus and the corresponding amino acid sequence of the isoamylase of the invention (SEQ ID NO: 4) shows about 54-55% homology to a deduced amino acid sequence based on the known DNA sequence above.

DEPR:
The invention also relates to a cloned DNA sequence encoding an enzyme with isoamylase activity derived from a strain of Sulfolobus. The DNA sequence may be derived from a strain of Sulfolobus acidocaldarius or Sulfolobus solfataricus.

DEPR:
The sequences may be the DNA sequences encoding an isoamylase from Sulfolobus acidocaldarius disclosed in Biochim. Biophys. Acta, 1291, p.177-181 (1996), it may be the DNA sequence from Sulfolobus solfataricus available in GeneBank under the Accession no. Y08256.

DEPR:
Cloning of the Sulfolobus acidocaldarius DNA sequence disclosed in Biochim. Biophys. Acta, 1291, p.177-181 (1996) is described below.

DEPR:
The isoamylase gene from Sulfolobus acidocaldarius was cloned by PCR. The primers were designed based on the sequence shown in the article of Table 1.

DEPR:
Cloned gene from Sulfolobus acidocaldarius was inserted at Hind III and XbaI site of pJSOHX and expression vector named pFSI82 was obtained. PFSI 82 was transformed to Saccharomyces cerevisiae YNG318. pFSI82 map is shown in FIG. 4.

DEPR:
The pH optimum of the Sulfolobus acidocaldarius isoamylase was determined. The Reaction was carried out at 70.degree. C. The checked pH was between pH 3.5-7.5. The pH optimum was determined to pH 5.5. The pH curve is shown in FIG. 5.

DEPR:
Temperature optimum of the Sulfolobus isoamylase was determined. The reaction was carried out at pH5.5 at between 40.degree.-90.degree. C. The optimum temperature was determined to be around 70.degree. C. The temperature curve is shown in FIG. 6.

DEPL:
Cloning of a DNA Sequence from a Strain of Sulfolobus

DEPL:
Cloning and Expression of **Sulfolobus acidocaldarius** Isoamylase Gene

DEPL:
Characterization of the Cloned **Sulfolobus acidocaldarius** Isoamylase

DETL:
identity Origins of isoamylase genes
DNA a.a. Pseudomonas amyloderamosa*1
50.4% 33.0% Pseudomonas sp. SMP1*2 50.4% 33.0% Flavobacterium sp.*3 54.0%
34.1% Flavobacterium odoratum*4 54.8% 36.6% **Sulfolobus** acidocaldarius*5 51.8%
54.2% **Sulfolobus** solfataricus*6 51.1% 55.0%
*1: Table 1, 1 *2: Table 1, 2 *3:
Table 1, 3 *4: Table 1, 4 *5: Table 1, 5 *6: GenBank: Accession number;
Y08256

DETL:
TABLE 1 1 Pseudomonas amyloderamosa
Biochim. Biophys. Acta, 1087, p. 309-315 (1990) 2 Pseudomonas sp. European
patent publication num- ber: EP 0 302 838 A2 3 Flavobacterium sp.
International patent publication number: WO 96/03513 4 Flavobacterium
odoratum Japanese patent publication num- ber: JP08023981-A 5 **Sulfolobus**
acidocaldarius Biochim. Biophys. Acta, 1291, p. 177-181 (1996)

DETL:
- - tggtacaact ggcagctcga cacgcgcaag cagcagtttc tggagttcgt gc - #gccagacg 1740
- - atctggtttc gcaagcagca tcggagcttc cggcgccgcc atttctgac cg - #gattgccc 1800
- - aacggcggaa ggcccccgacg cagtctggtg gcacctgagg gtcggcccat gc - #gccacgag 1860
- - gactggacca acccggagct gacggccttc ggactgctgc tgcacggcga cg - #ccattcag 1920
- - gggaccgacg agcacggacg accgtttcgc gacgacacgt ttctgattct gt - #tcaacaac 1980
- - ggcagcgaag ccgtgccggt cgtggtgccg gaggtatgct cctgtggcaa gc - #cgcaccac 2040
- - tgggaggtgg tcccggtgtt tcaacgcaat gtggagcccc ccacgtgcgc gc - #ccggcgag 2100
- - acgctgtcgc tcccgcccgg cgtgctgacg gtgctggtgg ccgtaccgcc gt - #tctcggat 2160
- - ggaaacacgg agccggcctg a - # - # 2181 - - - - <210> SEQ ID NO 4 <211>
LENGTH: 726 <212> TYPE: PRT <213> ORGANISM: Rhodothermus marinus - - <400>
SEQUENCE: 4 - - Met Ser His Ser Ala Gln Pro Val Thr Ser Va - #l Gln Ala Val
Trp Pro 1 5 - # 10 - # 15 - - Gly Arg Pro Tyr Pro Leu Gly Ala Thr Trp As - #p
Gly Leu Gly Val Asn 20 - # 25 - # 30 - - Phe Ala Leu Tyr Ser Gln His Ala Glu
Ala Va - #l Glu Leu Val Leu Phe 35 - # 40 - # 45 - - Asp His Pro Asp Asp Pro
Ala Pro Ser Arg Th - #r Ile Glu Val Thr Glu 50 - # 55 - # 60 - - Arg Thr Gly
Pro Ile Trp His Val Tyr Leu Pr - #o Gly Leu Arg Pro Gly 65 - #70 - #75 - #80
- - Gln Leu Tyr Gly Tyr Arg Val Tyr Gly Pro Ty - #r Arg Pro Glu Glu Gly 85 - #
90 - # 95 - - His Arg Phe Asn Pro Asn Lys Val Leu Leu As - #p Pro Tyr Ala Lys
Ala 100 - # 105 - # 110 - - Ile Gly Arg Pro Leu Arg Trp His Asp Ser Le - #u
Phe Gly Tyr Lys Ile 115 - # 120 - # 125 - - Gly Asp Pro Ala Gly Asp Leu Ser
Phe Ser Gl - #u Glu Asp Ser Ala Pro 130 - # 135 - # 140 - - Tyr Ala Pro Leu
Gly Ala Val Val Glu Gly Cy - #s Phe Glu Trp Gly Asp 145 1 - #50 1 - #55 1 -
#60 - - Asp Arg Pro Pro Arg Ile Pro Trp Glu Asp Th - #r Ile Ile Tyr Glu Thr
165 - # 170 - # 175 - - His Val Lys Gly Ile Thr Lys Leu His Pro Gl - #u Val
Pro Glu Pro Leu 180 - # 185 - # 190 - - Arg Gly Thr Tyr Leu Gly Leu Thr Cys
Glu Pr - #o Val Leu Glu His Leu 195 - # 200 - # 205 - - Lys Gln Leu Gly Val
Thr Thr Ile Gln Leu Le - #u Pro Val His Ala Lys 210 - # 215 - # 220 - - Val
His Asp Arg His Leu Val Glu Arg Gly Le - #u Arg Asn Tyr Trp Gly 225 2 - #30 2
- #35 2 - #40 - - Tyr Asn Pro Leu Cys Tyr Phe Ala Pro Glu Pr - #o Glu Tyr Ala
Thr Asn 245 - # 250 - # 255 - - Gly Pro Ile Ser Ala Val Arg Glu Phe Lys Me -
#t Met Val Arg Ala Leu 260 - # 265 - # 270 - - His Ala Ala Gly Phe Glu Val
Ile Val Asp Va - #l Val Tyr Asn His Thr 275 - # 280 - # 285 - - Gly Glu Gly
Gly Val Leu Gly Pro Thr Leu Se - #r Phe Arg Gly Ile Asp 290 - # 295 - # 300 -
- Asn Arg Ala Tyr Tyr Lys Ala Asp Pro Asn As - #n Pro Arg Phe Leu Val 305 3 -
#10 3 - #15 3 - #20 - - Asp Tyr Thr Gly Thr Gly Asn Thr Leu Asp Va - #l Gly
Asn Pro Tyr Val 325 - # 330 - # 335 - - Ile Gln Leu Ile Met Asp Ser Leu Arg
Tyr Tr - #p Val Thr Glu Met His 340 - # 345 - # 350 - - Val Asp Gly Phe Arg
Phe Asp Leu Ala Ala Al - #a Leu Ala Arg Glu Leu 355 - # 360 - # 365 - - Tyr
Asp Val Asp Met Leu Ser Thr Phe Phe Gl - #n Val Ile Gln Gln Asp 370 - # 375 -
380 - - Pro Val Leu Ser Gln Val Lys Leu Ile Ala Gl - #u Pro Trp Asp Val Gly
385 3 - #90 3 - #95 4 - #00 - - Pro Gly Gly Tyr Gln Val Gly His Phe Pro Tr -

#p Gln Trp Thr Glu Trp 405 - # 410 - # 415 - - Asn Gly Arg Tyr Arg Asp Ala Val Arg Arg Ph - #e Trp Arg Gly Asp Arg 420 - # 425 - # 430 - - Gly Leu Asn Gly Glu Phe Ala Thr Arg Phe Al - #a Gly Ser Ser Asp Leu 435 - # 440 - # 445 - - Tyr Glu Arg Ser Gly Arg Arg Pro Phe Ala Se - #r Ile Asn Phe Val Thr 450 - # 455 - # 460 - - Ala His Asp Gly Phe Thr Leu Glu Asp Leu Va - #l Ser Tyr Thr Lys Lys 465 4 - #70 4 - #75 4 - #80 - - His Asn Glu Ala Asn Leu Glu Gly Asn Arg As - #p Gly Met Asp Glu Asn 485 - # 490 - # 495 - - Tyr Ser Thr Asn Cys Gly Val Glu Gly Pro Th - #r Gln Asp Pro Ser Val 500 - # 505 - # 510 - - Leu Ala Cys Arg Glu Ala Leu Lys Arg Ser Le - #u Ile Ser Thr Leu Phe 515 - # 520 - # 525 - - Leu Ser Gln Gly Val Pro Met Leu Leu Gly Gl - #y Asp Glu Leu Ser Arg 530 - # 535 - # 540 - - Thr Gln His Gly Asn Asn Asn Ala Tyr Cys Gl - #n Asp Asn Glu Ile Ser 545 5 - #50 5 - #55 5 - #60 - - Trp Tyr Asn Trp Gln Leu Asp Thr Arg Lys Gl - #n Gln Phe Leu Glu Phe 565 - # 570 - # 575 - - Val Arg Gln Thr Ile Trp Phe Arg Lys Gln Hi - #s Arg Ser Phe Arg Arg 580 - # 585 - # 590 - - Arg His Phe Leu Thr Gly Leu Pro Asn Gly Gl - #y Arg Pro Arg Arg Ser 595 - # 600 - # 605 - - Leu Val Ala Pro Glu Gly Arg Pro Met Arg Hi - #s Glu Asp Trp Thr Asn 610 - # 615 - # 620 - - Pro Glu Leu Thr Ala Phe Gly Leu Leu Leu Hi - #s Gly Asp Ala Ile Gln 625 6 - #30 6 - #35 6 - #40 - - Gly Thr Asp Glu His Gly Arg Pro Phe Arg As - #p Asp Thr Phe Leu Ile 645 - # 650 - # 655 - - Leu Phe Asn Asn Gly Ser Glu Ala Val Pro Va - #l Val Val Pro Glu Val 660 - # 665 - # 670 - - Cys Ser Cys Gly Lys Pro His His Trp Glu Va - #l Val Pro Val Phe Gln 675 - # 680 - # 685 - - Arg Asn Val Glu Pro Pro Thr Cys Ala Pro Gl - #y Glu Thr Leu Ser Leu 690 - # 695 - # 700 - - Pro Pro Gly Val Leu Thr Val Leu Val Ala Va - #l Pro Pro Phe Ser Asp 705 7 - #10 7 - #15 7 - #20 - - Gly Asn Thr Glu Pro Ala 725 - - - - <210> SEQ ID NO 5 <211> LENGTH: 26 <212> TYPE: DNA <213> ORGANISM: Rodethermus marinus - - <400> SEQUENCE: 5 - - gtcagtagcc catggcacat tagcgc - # - # 26 - - - - <210> SEQ ID NO 6 <211> LENGTH: 24 <212> TYPE: DNA <213> ORGANISM: Rodethermus marinus - - <400> SEQUENCE: 6 - - ctcggccgga ctagatctgt cttc - # - # 24 - - - - <210> SEQ ID NO 7 <211> LENGTH: 39 <212> TYPE: DNA <213> ORGANISM: **Sulfolobus acidocaldarius** - - <400> SEQUENCE: 7 - - gtatatcaaa gcttatgaaa gatcgaccat taaagcctg - # - # 39 - - - - <210> SEQ ID NO 8 <211> LENGTH: 35 <212> TYPE: DNA <213> ORGANISM: **Sulfolobus acidocaldarius** - - <400> SEQUENCE: 8 - - ggttgtctag atcactggaa ctctatcctc ctgta - # - # 35 - - - - <210> SEQ ID NO 9 <211> LENGTH: 26 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <221> NAME/KEY: mutation <222> LOCATION: (0)...(0) <223> OTHER INFORMATION: Oligo 1 - - <400> SEQUENCE: 9 - - gatcaagctt ggaattcgct cgagct - # - # 26 - - - - <210> SEQ ID NO 10 <211> LENGTH: 26 <212> TYPE: DNA <213> ORGANISM: Artificial Sequence <220> FEATURE: <221> NAME/KEY: mutation <222> LOCATION: (0)...(0) <223> OTHER INFORMATION: Oligo 2 - - <400> SEQUENCE: 10 - - ctagagctcg agcgaattcc aagctt - # - # 26 - - - - <210> SEQ ID NO 11 <211> LENGTH: 140 <212> TYPE: PRT <213> ORGANISM: Flavobacterium sp. - - <400> SEQUENCE: 11 - - Phe Asn Pro Asn Lys Leu Leu Leu Asp Pro Ty - #r Ala Lys Ala Val His 1 5 - # 10 - # 15 - - Arg Gln Ile Asp Trp Asp Pro Ala Leu Phe Se - #r Tyr Asn Leu Gly Asp 20 - # 25 - # 30 - - Pro Asp Ser Arg Asn Asp Asp Asp Ser Ala Pr - #o His Met Met Leu Gly 35 - # 40 - # 45 - - Val Val Ile Asn Pro Phe Phe Asp Trp Asp Gl - #y Asp Lys Leu Pro Arg 50 - # 55 - # 60 - - Ile Pro Tyr His Lys Ser Val Ile Tyr Glu Al - #a His Val Lys Gly Leu 65 - #70 - #75 - #80 - - Thr Gln Leu His Pro Glu Val Pro Glu Gly Al - #a Ala Arg Tyr Tyr Ala 85 - # 90 - # 95 - - Gly Val Ala His Pro Ala Val Ile Ser His Le - #u Gln Lys Leu Gly Ile 100 - # 105 - # 110 - - Thr Ala Ile Glu Leu Met Pro Val His Gln Ph - #e Val Asn Asp Gly Ile 115 - # 120 - # 125 - - Leu Gln Asp Lys Gly Leu Asn Asn Tyr Trp Gl - #y Tyr 130 - # 135 - # 140 - - - - <210> SEQ ID NO 12 <211> LENGTH: 143 <212> TYPE: PRT <213> ORGANISM: Flavobacterium devorans - - <400> SEQUENCE: 12 - - Phe Asn Pro Asn Lys Val Leu Leu Asp Pro Ty - #r Ala Arg Lys Leu Phe 1 5 - # 10 - # 15 - - Gly Glu Ile Lys Trp Thr Asp Ala Leu His Gl - #y Tyr Gln Ile Arg Ser 20 - # 25 - # 30 - - Lys Lys Glu Asp Leu Ser Phe Asp Lys Arg As - #p Ser Ala Ala Ala Met 35 - # 40 - # 45 - - Pro Lys Ala Val Val Val Asp Asp His Phe As - #p Trp Ser Arg Asp Val 50 - # 55 - # 60 - - Lys Pro Asn Thr Pro Trp Ser Glu Thr Val Il - #e Tyr Glu Ala His Val 65 - #70 - #75 - #80 - - Lys Gly Leu Thr Lys Leu Met Glu Leu Val Pr - #o Pro Arg Glu Arg Gly 85 - # 90 - # 95 - - Thr Tyr Ala Gly Leu Gly His Pro Ala Val Il - #e Lys His Leu Lys Arg 100 - # 105 - # 110 - - Ile Gly Val Thr Ala Ile Glu Leu Leu Pro Il - #e His Ser Phe Thr Gln 115 - # 120 - # 125 - - Asp Arg Phe Leu Gln Glu Lys Gly Leu Arg As - #n Tyr Trp Gly Tyr 130 - # 135 - # 140 - - - - <210> SEQ ID NO 13 <211> LENGTH: 143 <212> TYPE: PRT <213> ORGANISM: Xanthomonas camperstris - - <400> SEQUENCE: 13 - - Phe Asn Pro Asn Lys Val Leu Leu Asp Pro Ty - #r Ala Arg Glu Leu Asp 1 5 - # 10 - # 15 - - Gly Asp Leu Val Trp Ala Asp Glu Leu Tyr Gl - #y Tyr Thr Val Gly His 20 - # 25 - # 30 - - Pro Asp Gly

Asp Leu Ser Phe Asp Glu Arg As - #p Ser Ala Pro Phe Met 35 - # 40 - # 45 - -
Pro Lys Cys Val Val Val Glu Asp Thr Tyr As - #p Trp Glu Asp Asp Ala 50 - # 55
- # 60 - - Arg Leu Leu Lys Pro Trp Asn Glu Thr Val Il - #e Tyr Glu Thr His Val
65 - #70 - #75 - #80 - - Arg Gly Tyr Thr Met Arg Asn Ala Gln Val Pr - #o Glu
Ala Val Arg Gly 85 - # 90 - # 95 - - Thr Phe Ala Gly Leu Ala Gln Pro Ser Val
Le - #u Gln Tyr Ile Lys Asp 100 - # 105 - # 110 - - Leu Gly Ile Thr Ala Val
Glu Leu Leu Pro Va - #l His Ala Tyr Leu Asp 115 - # 120 - # 125 - - Asp Gln
His Leu Leu Asp Lys Gly Leu Arg As - #n Tyr Trp Gly Tyr 130 - # 135 - # 140 -
- - - <210> SEQ ID NO 14 <211> LENGTH: 143

USPT

US-CL-CURRENT: 424/93.43,435/262 ,435/886

US-PAT-NO: 6066492
DOCUMENT-IDENTIFIER: US 6066492 A
TITLE: Microorganism capable of degrading polylactic acid resin and method of degrading polylactic acid resin using said microorganism
DATE-ISSUED: May 23, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tokiwa; Yutaka	Tsuchiura	N/A	N/A	JPX
Jikuya; Hiroyuki	Tsukuba	N/A	N/A	JPX
Nagai; Naoko	Kameoka	N/A	N/A	JPX

US-CL-CURRENT: 435/253.5,424/93.43 ,435/262 ,435/886
ABSTRACT:
A microorganism belonging to the genus Staphylococcus or the genus Streptomyces which is capable of degrading a polylactic acid resin. A method of degrading a polylactic acid resin including a step of culturing a microorganism capable of degrading a polylactic acid resin in a medium containing a polylactic acid resin. In particular, the microorganisms Streptomyces violaceusniger FERM BP-6110 and Streptomyces cyaneus FERM BP-6111 are used.
6 Claims, 7 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

DETL:
TABLE 1-3 ______ Genera of Bacteria ______ Cardiobacterium Thiobacillus Streptobacillus **Sulfolobus** Calymmatobacterium Thiobacterium BACTEROIDACEAE Macromonas Bacteroides Thiovulum Fusobacterium Thiospira Leptotrichia SIDEROCAPSACEAE Desulfovibrio Siderocapsa Butyrivibrio Naumaniella Succinivibrio Ochrobium Succinimonas Siderococcus Lachnospira METHANOBACTERIACEAE Selenomonas Methanobacterium NEISSERIACEAE Methanosarcina Neisseria Methanococcus Branhamella MICROCOCCACEAE Moroxella Micrococcus Acinetobacter Staphylococcus Paracoccus Planococcus Lampropedia STREPTOCOCCACEAE VEILLONELLACEAE Streptococcus Veillonella Leuconostoc Acidaminococcus Pediococcus Megasphaera Aerococcus NITROBACTERACEAE Gemella Nitrobacter PERPTOCOCCACEAE Nitrospina Peptococcus Nitrococcus Peptostreptococcus Nitrosomonas Ruminococcus Nitrosospira Sarcina Nitrosococcus Nitrosolobus ______

DETL:
TABLE 3 ______ Strain Staphylococcus (FERM BP-6108) ______ Colony Morphology Spherule, cream color, 1 mm in diameter Gram Staining + Spore Formation - Motility - Aerial Mycelium - Growth at: 30.degree. C. + 37.degree. C. + 50.degree. C. - Catalase + Oxidase - OF Test + Glucose + Maltose + Mannose + Raffinose - **Trehalose** + Fructose + Xylose - Sucrose + Mannitol + Lactose - Melibiose - ______

DETL:
Spore Formation - Motility - Aerial Mycelium - Growth at: 30.degree. C. - 37.degree. C. - 50.degree. C. + Catalase + Oxidase - OF Test + Glucose + Maltose + Mannose + Raffinose - **Trehalose** - Fructose + Xylose - Sucrose + Mannitol - Lactose + Melibiose - ______

USPT

US-CL-CURRENT: 435/14,435/25 ,435/4 ,536/26.24

US-PAT-NO: 6046018
DOCUMENT-IDENTIFIER: US 6046018 A
TITLE: Highly sensitive method for assaying chiro-inositol and compositions for the assay
DATE-ISSUED: April 4, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kozuma; Takuji	Shizuoka	N/A	N/A	JPX
Takahashi; Mamoru	Shizuoka	N/A	N/A	JPX

US-CL-CURRENT: 435/26,435/14 ,435/25 ,435/4 ,536/26.24

ABSTRACT:
The present invention relates to an assay method of chiroinositol which comprises reacting a specimen containing chiroinositol with

1) a dehydrogenase, which catalyses at least reversible reaction with a substrate of chiroinositol in the presence of a coenzyme selected from NAD(P)s and a coenzyme selected from thio-NAD(P)s,

2) A1 and

3) B1

to form cycling reaction of the formula ##STR1## wherein a product is a compound, from which 2 or 4 hydrogen atoms are deleted from chiroinositol, A1 is NAD(P)s or thio-NAD(P)s, A2 is a reduced form of A1, B1 is a reduced form of NAD(P)s in case of A1 being thio-NAD(P)s or a reduced form of thio-NAD(P)s in case of A1 being NAD(P)s and B2 is an oxidized form of B1, and determining an amount of converted A2 or B1 by the said reaction, and a composition for assay of chiroinositol. Chiroinositol can be assayed by accurate, simple, low price and high sensitive method.
5 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

BSPR:
Species of this strain in Bacillus is identified as follows. According to Bergey's Manual of Systematic Bacteriology, Vol. 2, Bacillus species, which grow at high temperature (at 50.degree. C.), are known 9 species of B. acidocaldarius, B. subtilis, B. badius, B. brevis, B. coagulans, B. licheniformis, B. petntothenticus, B. schegelli and B. stearothermophilus. Among them, the species which grow under anaerobic condition are only known two species, i. e. coagulans (hereinafter sometimes designates as "C") and B. licheniformis (hereinafter sometimes designates as "L"). Comparison with taxonomical properties of C, L and the present strain are shown as follows.

BSTL:
____ GC molar ratio % : 41.9% (HPLC) Major isoprenoid quinone : MK-7 Gram's stain + KOH reaction - Capsule formation - Acid-fast stain - OF test (Hugh-Leifson) NT OF test (nitrogen source : NH.sub.4 H.sub.2 PO.sub.4) F Aerobic growth + Anaerobic growth + Growth temperature at 70.degree. C. - 60.degree. C. + 37.degree. C. + 30.degree. C. - Halotolerant 0% + 3% + 5% - Growth pH 5.6 - 6.2 - 9.0 + Gelatin hydrolysis + Starch hydrolysis (+) Casein hydrolysis - Esculin hydrolysis + Tyrosine hydrolysis - Arginine hydrolysis - Cellulose hydrolysis - Catalase production + Oxidase production + Lecithinase production - Urease production (SSR) - Urease production (Chris) - Indole production - H.sub.2 S production (detected by lead acetate paper) - Acetoin production (K.sub.2 HPO.sub.4) - Acetoin Production (NaCl) - MR test - Nitrate reduction test (gas formation) - (NO.sub.2 - detection) - (NO.sub.3 - detection) + Utilization on Simmons medium Citrate - Malate - Maleate - Malonate - Propionate - Gluconate - Succinate - Utilization on Christensen medium Citrate + Malate - Maleate - Malonate - Propionate + Gluconate - Succinate - Gas formation from glucose

- Acid formation from various sugars Adnitol - L (+) arabinose - Cellobiose + Dulcitol - Meso-erythritol - Fructose + Fucose + Galactose + Glucose + Glycerin + Inositol + Inulin + Lactose + Maltose + Mannitol + Mannose + Melezitose - Melibiose + Raffinose - Rhamnose + D-ribose + Salicin + L-sorbose - Sorbitol - Starch + Saccharose + **Trehalose** + Xylose -

USPT

US-CL-CURRENT: 435/183,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ,536/23.2 ,536/23.7

US-PAT-NO: 6027918
DOCUMENT-IDENTIFIER: US 6027918 A
TITLE: Recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharide
DATE-ISSUED: February 22, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/69.2,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ,536/23.2 ,536/23.7

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of **trehalose** on an industrial scale and in a satisfactorily-high yield.
13 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

TTL:
Recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharide

ABPL:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of **trehalose** on an industrial scale and in a satisfactorily-high yield.

BSPR:
The present invention relates to a recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:
Trehalose is a disaccharide which consists of 2 glucose molecules that are linked together with their reducing groups, and, naturally, it is present in fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, **trehalose** does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can advantageously sweeten food products without fear of causing unsatisfactory coloration and deterioration. **Trehalose**, however, could not have been readily prepared in a desired amount by conventional production methods, so that it has not scarcely been used for sweetening food products.

BSPR:
Conventional production methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other using a multi-enzymatic system where several enzymes are allowed to act on saccharides. The former, as

disclosed in Japanese Patent Laid-Open No.154,485/75, is a method which comprises growing microorganisms such as bacteria and yeasts in nutrient culture media, and collecting **trehalose** mainly from the proliferated cells. The latter, as disclosed in Japanese Patent Laid-Open No.216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and **trehalose**-phosphorylases to act on maltose, and recovering the formed **trehalose** from the reaction system. The former facilitates the growth of microorganisms, but has a demerit that the content in the microorganisms is at most 15 w/w %, on a dry solid basis (d.s.b.). Although the latter can readily separate **trehalose**, it is theoretically difficult to increase the **trehalose** yield by allowing such phosphorylases to act on substrates at a considerably-high concentration because the enzymatic reaction in itself is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

BSPR:
In view of the foregoing, the present inventors energetically screened enzymes which form saccharides having a **trehalose** structure from amylaceous saccharides, and have found that microorganisms such as those of the genera Rhizobium and Arthrobacter produce an absolutely novel enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. They disclosed such an enzyme in Japanese Patent Application No.349,216/93. At almost the same time, they also found that these non-reducing saccharides are nearly quantitatively hydrolyzed into **trehalose** and glucose and/or maltooligosaccharides by other enzymes produced from the same microorganisms of the genera Rhizobium and Arthrobacter.

BSPR:
It was found that the enzymes produced from the aforesaid microorganisms have an optimum temperature of about 40.degree. C., and have some difficulties in their thermostability when actually used to produce **trehalose**. It is recognized in this field that a recommendable temperature in the saccharification reaction of starch or amylaceous saccharides is one which exceeds 55.degree. C. because bacterial contamination will occur at a temperature of 55.degree. C. or lower and decreasing the pH of the reaction mixtures and inactivating the enzymes used. Thus, a relatively-large amount of substrates remain intact. While the use of enzymes with a poor thermostability, a great care should be taken to control the pH, and, when the pH level lowers to an extremely low level, alkalis should be added to reaction mixtures to increase the pH level as quickly as possible.

BSPR:
In view of the foregoing, the present inventors screened thermostable enzyme with a satisfactory activity and have found that enzymes produced from microorganisms of the genus **Sulfolobus** including **Sulfolobus acidocaldarius** (ATCC 33909) are not substantially inactivated even when incubated at a temperature exceeding 55.degree. C., and they efficiently release **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. These microorganisms, however, are not sufficient in the enzyme productivity, and this requires a relatively-large scale culture to industrially produce **trehalose** from those non-reducing saccharides.

BSPR:
It is an object of the present invention to provide a recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 by using the recombinant DNA technology.

BSPR:
It is another object of the present invention to provide an enzymatic conversion method for releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:

The sixth object of the present invention is attained by an enzymatic conversion method of non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, which contains a step of allowing the recombinant thermostable enzyme to act on the non-reducing saccharides to release **trehalose**.

BSPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3;

DRPR:
FIG. 1 is a figure of the optimum temperature of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 2 is a figure of the optimum pH of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 3 is a figure of the thermostability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 4 is a figure of the pH stability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The recombinant thermostable enzyme according to the present invention releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 without substantial inactivation even when allowed to react at a temperature exceeding 55.degree. C.

DEPR:
According to the present enzymatic conversion method converts non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 into saccharide compositions containing **trehalose** and glucose and/or maltooligosaccharides.

DEPR:
The present invention has been made based on the finding of a novel enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. Such an enzyme is obtainable from cultures of microorganisms of the species **Sulfolobus acidocaldarius** (ATCC 33909). The present inventors isolated such an enzyme by using in combination a various purification methods comprising column chromatography as a main technique, studied their properties and features, and revealed that the reality is a polypeptide with the following physicochemical properties:

DEPR:
The followings are the explanations of the experiments conducted to reveal the physicochemical properties of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909):

DEPR:
Into 500-ml flasks were poured 100 ml aliquots of a liquid culture medium containing 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min. After cooling the flasks a seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each liquid culture medium in each flask, followed by the incubation at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was poured into a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the first seed culture into the sterilized liquid

culture medium in the fermenter, and culturing the microorganisms at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was poured into a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the second seed culture into the sterilized liquid culture medium, and culturing the microorganisms at 75.degree. C. for 42 hours under an aeration condition of 100 L/min.

DEPR:
The results in Table 1 show that the purified enzyme nearly quantitatively releases **trehalose** and glucose or maltooligosaccharides from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, but does not substantially act on maltooligosaccharides having a degree of glucose polymerization of at least 3. These facts indicate that the purified enzyme specifically acts on non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, and specifically hydrolyzes the glycosidic linkages between **trehalose**- and glycosyl-residues. Such an enzyme has not been reported and it can be estimated to have a novel enzymatic pathway.

DEPR:
A chromosomal DNA of **Sulfolobus acidocaldarius** (ATCC 33909) was screened by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequences in SEQ ID NOs:3 and 4, and this yielded a DNA fragment having a base sequence (SEQ ID NO:2) from the 5'-terminus consisting of about 1,700 base pairs. The base sequence of the thermostable enzyme was decoded and revealing that it consists of 556 amino acids and has a partial amino acid sequence from the N-terminal in SEQ ID NO:1.

DEPR:
To 500-ml flasks were placed about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to a pH 3.0 by the addition of sulfate. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each flask, incubated at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a seed culture. About 5 L of a fresh preparation of the same liquid nutrient culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to a pH 3.0, and inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min.

DEPR:
As a control, a seed culture of Escherichia coli XLI-Blue strain or **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into a fresh preparation of the same liquid culture medium but free of ampicillin. In the case of culturing **Sulfolobus acidocaldarius** (ATCC 33909), it was cultured and treated similarly as above except that the initial pH of the nutrient culture medium and the culturing temperature were respectively set to 3.0 and 75.degree. C. Assaying the resultant enzymatic activity, one L culture of **Sulfolobus acidocaldarius** (ATCC 33909) yielded about 2 units of the thermostable enzyme, and the yield was significantly lower than that of transformant SU18. Escherichia coli XLI-Blue strain used as a host did not form the thermostable enzyme.

DEPR:
Thereafter, the recombinant thermostable enzyme produced by the transformant SU18 was purified similarly as in Experiments 1 and 2 and examined for properties and features and revealing that it has substantially the same physicochemical properties of the thermostable enzyme from **Sulfolobus acidocaldarius** (ATCC 33909) because (i) the recombinant thermostable enzyme has a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and an isoelectric point of about 5.6-6.6 on isoelectrophoresis, and (ii) it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These results indicate that the present thermostable enzyme can be prepared by the recombinant DNA technology with a

significantly improved yield.

DEPR:
Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of about 1,700 base pairs in SEQ ID NO:5. An amino acid sequence that could be estimated from the base sequence was in SEQ ID NO:5, and it was compared with the partial amino acid sequences in SEQ ID NOs:3 and 4, and revealing that the partial amino acid sequence in SEQ ID NO:3 corresponded to that positioning from 1 to 30 in SEQ ID NO:5, and that in SEQ ID NO:4 corresponded to that positioning from 301 to 319 in SEQ ID NO:5. These results indicate that the present recombinant thermostable enzyme has the amino acid sequence from the N-terminal in SEQ ID NO:1, and, in the case of the DNA derived from **Sulfolobus acidocaldarius** (ATCC 33909), the amino acid sequence is encoded by the base sequence from the 5'-terminus in SEQ ID NO:2.

DEPR:
As is explained in the above, the thermostable enzyme, which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, was found as a result of the present inventors' long-term research. The thermostable enzyme has distinct physicochemical properties from those of other conventional enzymes. The present invention is to produce the thermostable enzyme by using the recombinant DNA technology. The present recombinant thermostable enzyme, its preparation and uses will be explained in detail with reference to the later described Examples.

DEPR:
The recombinant thermostable enzyme as referred to in the present invention means thermostable enzymes in general which are preparable by the recombinant DNA technology and capable of releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. Generally, the recombinant thermostable enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence from the N-terminal as shown in SEQ ID NO:1, and homologous ones to it can be mentioned. Variants having amino acid sequences homologous to the one in SEQ ID NO:1 can be obtained by replacing one or more amino acids in SEQ ID NO:1 with other amino acids without substantially altering the inherent physicochemical properties. Although even when used the same DNA and it also depends on hosts into which the DNA is introduced, the ingredients and components of nutrient culture media for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have the inherent physicochemical properties but defect one or more amino acids in SEQ ID NO:1, or those which have one or more amino acids added newly to the N-terminal after the DNA expression as the result of the modification of intracellular enzymes of the hosts. Such variants can be used in the present invention as long as they have the desired physicochemical properties.

DEPR:
The DNA usable in the present invention includes those are derived from natural resources and those which are artificially synthesized as long as they have the aforesaid base sequences. The natural resources for the DNA according to the present invention are, for example, microorganisms of the genus **Sulfolobus such as Sulfolobus acidocaldarius** (ATCC 33909), and from which genes containing the present DNA can be obtained. The aforementioned microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or .beta.-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, when treated the cells with an ultrasonic disintegrator, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or with freezing and thawing method. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment generally used in this field. To artificially synthesize the present DNA, it can be chemically synthesized by using the base sequence in SEQ ID

NO:2, or can be obtained in a plasmid form by inserting a DNA, which encodes the amino acid sequence in SEQ ID NO:1, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the objective DNA from the cells.

DEPR:
The recombinant DNA thus obtained can be introduced into appropriate host microorganisms including Escherichia coli and those of the genus Bacillus as well as actinomyces and yeasts. In the case of using Escherichia coli as a host, the DNA can be introduced thereinto by culturing the host in the presence of the recombinant DNA and calcium ion, while in the case of using a microorganism of the genus Bacillus as a host the competent cell method and the colony hybridization method can be used. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, and selecting the objective transformants which release **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

DEPR:
The transformants thus obtained intra- and extra-cellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid culture media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with small amounts of amino acids and vitamins can be used in the invention. Examples of the carbon sources are saccharides such as unprocessed starch, starch hydrolysate, glucose, fructose, sucrose and **trehalose**. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia and salts thereof, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor, and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 20-65.degree. C. and a pH of 2-9 for about 1-6 days under aerobic conditions by the aeration-agitation method. Such cultures can be used intact as a crude enzyme, and, usually, cells in the cultures may be disrupted prior to use with ultrasonic and/or cell-wall lysis enzymes, followed by separating the thermostable enzyme from intact cells and cell debris by filtration and/or centrifugation and purifying the enzyme. The methods to purify the enzyme include conventional ones in general. From cultures intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

DEPR:
As is described above, the recombinant thermostable enzyme according to the present invention has a specific feature of releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 even when allowed to act on them at a temperature exceeding 55.degree. C. The **trehalose** thus obtained has a satisfactorily-mild and high-quality sweetness as well as an adequate viscosity and moisture-retaining ability, and, as a great advantageous feature, they can sweeten food products without fear of causing unsatisfactory coloration and deterioration because they have no reducing residue within their molecules. With these features a variety of amylaceous saccharides, which have been put aside because of their reducibilities, can be converted into saccharides which have a satisfactory handleability, usefulness, and no substantial reducibility or extremely-reduced reducibility.

DEPR:
Explaining now the conversion method in more detail, non-reducing saccharides having a **trehalose** structure and a degree of glucose polymerization of at least 3 such as .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose. These non-reducing saccharides can be

obtained by allowing a non-reducing saccharide-forming enzyme as disclosed in Japanese Patent Application No.349,216/93, applied by the present applicant and Japanese Patent Application Serial No.10046601, titled "Thermostable non-reducing saccharide-forming enzyme, its preparation and uses", applied by the same applicant on Jun. 24, 1994, to act on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 which are prepared by treating starch or amylaceous saccharides such as amylopectin and amylose with acids and/or amylases. These reducing saccharides usable as a substrate for the non-reducing saccharide-forming enzyme usually contain one or more maltooligosaccharides having a degree of glucose polymerization of at least 3, for example, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. As is described in "Handbook of Amylases and Related Enzymes", 1st edition (1988), edited by The Amylase Research Society of Japan, published by Pergamon Press plc, Oxford, England, .alpha.-amylase, maltotetraose-forming amylase, maltopentaose-forming amylase and maltohexaose-forming amylase are especially useful to prepare the reducing amylaceous saccharides used in the present invention, and, the use of any one of these amylases facilitates the production of mixtures of amylaceous saccharides rich in reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 in a considerably-high yield. If necessary, the combination use of the amylases and starch debranching enzymes such as pullulanase and isoamylase can increase the yield of the reducing amylaceous saccharides used as the substrate for the present recombinant thermostable enzyme. Non-reducing saccharides can be obtained in a desired amount by coexisting such a non-reducing saccharide-forming enzyme in aqueous solutions containing one or more reducing amylaceous saccharides up to 50 w/w %, and, usually, incubating the mixture solution at a temperature of 40-85.degree. C. and a pH of about 4-8 until the non-reducing saccharides are produced.

DEPR:
In the enzymatic conversion method according to the present invention, the present recombinant thermostable enzyme is generally allowed to coexist in an aqueous solution containing one or more of the above non-reducing saccharides as a substrate, followed by the enzymatic reaction at a prescribed temperature and pH until a desired amount of **trehalose** is formed. Although the enzymatic reaction proceeds even at a concentration of about 0.1 w/w %, d.s.b., of a substrate, a concentration of 2 w/w % or higher, d.s.b., preferably, in the range of 5-50 w/w %, d.s.b., of a substrate can be satisfactorily used when used the present conversion method in an industrial-scale production. The temperature and pH used in the enzymatic reaction are set to within the range of which does not inactivate the recombinant thermostable enzyme and allows the enzyme to effectively act on substrates, i.e. a temperature of higher than 55.degree. C. but not higher than 85.degree. C., preferably, a temperature in the range of about 56-70.degree. C., and a pH of 4-7, preferably, a pH in the range of about 5-6. The amount and reaction time suitable for the present recombinant thermostable enzyme are chosen depending on the enzymatic reaction condition. Thus, the present recombinant thermostable enzyme effectively converts non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 into **trehalose** and glucose and/or maltooligosaccharides, e.g. the conversion rate increases up to about 99% when the enzyme acts on .alpha.-maltotriosyltrehalose. When either of amylases is allowed to act on starch hydrolysates in combination with the non-reducing saccharide-forming enzyme and the present thermostable enzyme, non-reducing saccharides are formed along with **trehalose** and glucose and/or maltooligosaccharides. Thus, saccharide compositions rich in **trehalose** are efficiently formed in a relatively-high yield.

DEPR:
The **trehalose** and compositions containing it have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
The purified enzyme was assayed for properties and features by the method in Experiment 2 and revealing that it had a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and a pI of about 5.6-6.6 on

isoelectrophoresis, and was not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These physicochemical properties were substantially the same as those of the thermostable enzyme from a donor microorganism of **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
To 500-ml flasks were added about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water. The flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled and adjusted its pH to 3.0 by the addition of sulfuric acid. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated to the flasks, and cultured at 75.degree. C. for 24 hours under a rotatory shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to pH 3.0, inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min to obtain a second seed culture. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium in a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and cultured under aeration and agitation conditions of 100 L/min for 42 hours.

DEPR:
Fractions with an enzymatic activity eluted at about 0.8 M ammonium sulfate were collected, pooled, dialyzed for 16 hours against 10 mM Tris-HCl buffer (pH 8.5) containing 0.2 M sodium chloride, and centrifuged at 10,000 rpm for 30 min to remove insoluble substances. The resultant supernatant was fed to a column packed with about 350 ml of "TOYOPEARL.RTM. HW-55", a gel for gel chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 0.2 M sodium chloride. Fractions with an enzymatic activity were collected from the eluate, pooled, and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) for 16 hours. The dialyzed solution was centrifuged to remove insoluble substances, and the supernatant was subjected to hydrophobic column chromatography using "MONO Q", a gel for ion-exchange chromatography commercialized by Pharmacia LKB Uppsala, Sweden, followed by feeding to the column with a linear gradient buffer ranging from 0 M to 0.2 M of sodium chloride in 10 mM Tris-HCl buffer (pH 8.5). The fractions eluted at about 0.1 M sodium chloride were collected and pooled for the production of **trehalose**. The purified non-reducing saccharide-forming enzyme thus obtained had a specific activity of about 81 units/mg protein, and the yield was about 0.24 units per one L of the culture.

DEPR:
The syrup had a relatively-low DE (dextrose equivalent) and contained 71.0 w/w % a **trehalose**, 2.9 w/w % glucosyltrehalose, 1.0 w/w % maltosyltrehalose, 4.9 w/w % glucose, 10.5 w/w % maltose, 8.2 w/w % maltotriose and 1.5 w/w % maltotetraose and higher maltooligosaccharides, d.s.b. The product, having a mild and moderate sweetness as well as an adequate viscosity and moisture-retaining ability, can be satisfactorily used in compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
A syrupy product in Example B-1 was column chromatographed using a strong-acid cation exchange resin to increase the **trehalose** content. The procedures were as follows: Four jacketed-stainless steel columns, 5.4 cm in diameter and 5 m in length each, were packed to homogeneity with "XT-1016 (Na.sup.+ -form)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, which had been previously suspended in water, and cascaded in series to give a total column length of 20 m. The columns were fed with the syrupy product, adequately diluted with water, in a volume of about 5 v/v % to the resin and at an inner column temperature of 55.degree. C., and fed with 55.degree. C. hot water at an SV (space velocity) 0.13 to elute saccharide components. Fractions rich in **trehalose** were collected,

pooled, concentrated, dried in vacuo and pulverized to obtain a powdery product containing about 97 w/w % **trehalose** in a yield of about 55 w/w % to the material, d.s.b.

DEPR:
A fraction rich in **trehalose** obtained by the method in Example B-2 was concentrated into an about 75 w/w % solution which was then transferred to a crystallizer, and crystallized under gently stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was sprayed downward from a nozzle equipped on the upper part of a spraying tower at a pressure of about 150 kg/cm.sup.2 while an about 85.degree. C. hot air was blowing downward from the upper part of the spraying tower, and the formed crystalline powder was collected on a wire-netting conveyer provided on the basement of the drying tower and gradually conveyed out of the spraying tower while an about 45.degree. C. hot air was blowing to the crystalline powder from under the conveyer. The crystalline powder thus obtained was transferred to an ageing tower and aged for 10 hours in a stream of about 40.degree. C. hot air to complete the crystallization and drying. Thus, a powdery hydrous crystalline **trehalose** was obtained in a yield of about 90 w/w % to the material, d.s.b.

DEPR:
Conversion into powdery product containing crystalline **trehalose**

DEPR:
Tapioca starch was dissolved in water into a 36 w/w % suspension which was then admixed with 0.1 w/w % calcium carbonate. The mixture was adjusted to pH 6.0, admixed with 0.2 w/w a of "TERMAMYL 60L", an .alpha.-amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, to starch, d.s.b., and enzymatically reacted at 95.degree. C. for 15 min to gelatinize and liquefy the starch. The mixture was autoclaved at 120.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 58.degree. C., adjusted to pH 5.2, admixed with 2,000 units/g starch, d.s.b., of an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 2.5 units/g starch, d.s.b., of a thermostable enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.s.b, of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 72 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 50.degree. C., admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered, decolored in usual manner with an activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 75.5 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was concentrated into an about 84 w/w % solution which was then transferred to a crystallizer, admixed with about 2 w/w %, d.s.b., of hydrous crystalline **trehalose** as a seed crystal, and crystallized under gentle stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was distributed to plastic plane vessels, allowed to stand at ambient temperature for 3 days to solidify and age the contents. Thereafter, the formed blocks were removed from the vessels, powdered by a pulverizer to obtain a solid product containing hydrous crystalline **trehalose** in a yield of about 90 w/w % to the material starch, d.s.b.

DEPR:
Potato starch was suspended in water into a 6 w/w % suspension which was then admixed with 0.01 w/w % "NEO-SPITASE", an .alpha.-amylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, adjusted to pH 6.2, and subjected to an enzymatic reaction at a temperature of 85-90.degree. C. for one hour to gelatinize and liquefy the starch. The mixture was heated at 120.degree. C. for 10 min to inactivate the remaining enzyme, cooled to 60.degree. C., adjusted to pH 5.5, admixed with 500 units/g starch, d.s.b., of "PROMOZYME 200L", a pullulanase specimen commercialized by Novo Nordisk

Bioindustri A/S, Copenhagen, Denmark, 3.0 units/g starch, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered, decolored in usual manner with an activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 79.3 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was column chromatographed similarly as in Example B-2 except that "C6000", commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used a strong-acid cation exchange resin in Na.sup.+ -form, followed by collecting a fraction containing about 95 w/w % **trehalose,** d.s.b. The fraction was concentrated up to about 75 w/w % and crystallized similarly as in Example B-4 to obtain a massecuite in the from of block which was then pulverized to obtain a powdery product containing hydrous crystalline **trehalose** in a yield of about 70 w/w % to the material starch, d.s.b.

DEPR:
One part by weight of "EX-I", an amylose product commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was dissolved by heating in 15 parts by weight of water, and the solution was adjusted to 65.degree. C. and pH 5.5, admixed with 2.0 units/g amylose, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a) and 6.0 units/g amylose, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-2, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was incubated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and pH 5.0, admixed with 10 units/g amylose, d.s.b., "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and incubated for another 40 hours. The newly formed reaction mixture was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered in usual manner, decolored with an activated charcoal, deionized and purified with an ion exchanger, and concentrated up to give a concentration of about 60 w/w % to obtain a syrupy product containing 82.2 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was similarly as in Example B-5 subjected to column fractionation to obtain a fraction containing 98 w/w % **trehalose,** d.s.b., which was then concentrated by heating under a reduced pressure up to give a concentration of about 85 w/w %. To the concentrate was added about 2 w/w % anhydrous crystalline **trehalose** as a seed, followed by mixing the resultant at 120.degree. C. for 5 min under stirring conditions. The resultant mixture was distributed to plastic plain vessels, and crystallized by drying in vacuo at 100.degree. C. Thereafter, products in the form of a block were removed from the vessels, pulverized with a cutter to obtain a solid product, which contained anhydrous crystalline **trehalose** and had a moisture content of about 0.3 w/w % and a crystallization percentage of about 70 w/w %, in a yield of about 70% to the material amylose, d.s.b.

DEPR:
Anhydrous crystalline **trehalose** absorbs moisture from anhydrous substances to convert into hydrous crystalline **trehalose,** and because of this the product rich in such anhydrous crystalline **trehalose** is useful as a desiccant to dehydrate compositions such as food products, cosmetics and pharmaceuticals, and their materials and intermediates. The product with a mild and high-quality sweetness can be arbitrarily incorporated into compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, diluent and excipient.

DEPR:

As is described above, the present invention is based on the finding of a novel thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The present invention is to explore a way to produce such a thermostable enzyme in an industrial scale and in a relatively-high efficiency by the recombinant DNA technology. The present conversion method using the recombinant thermostable enzyme readily converts non-reducing amylaceous saccharides, which have a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, into **trehalose** and glucose and/or maltooligosaccharides without fear of causing bacterial contamination. The **trehalose** has a mild and high-quality sweetness, and, because it has no reducing residue within the molecule, it can be advantageously incorporated into compositions in general such as food products, cosmetics and pharmaceuticals without fear of causing unsatisfactory coloration and deterioration. The present recombinant thermostable enzyme is one which has a revealed amino acid sequence, so that it can be used freely in the preparation of **trehalose** that is premised to be used in food products and pharmaceuticals.

DEPC:
Conversion into syrupy product containing **trehalose**

DEPC:
Conversion into syrupy product containing **trehalose**

DEPC:
Conversion into powdery product containing **trehalose**

DEPC:
Conversion into powder product containing crystalline **trehalose**

DEPC:
Conversion into powdery product containing anhydrous crystalline **trehalose**

DEPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3;

DETL:
TABLE 1

Saccharide in Elution time on HPLC Composition Substrate reaction mixture (min) (%)

.alpha.-Glucosyltrehalose **Trehalose** 27.4 7.2 Glucose 33.8 3.9 .alpha.-Glucosyltrehalose 23.3 88.9 .alpha.-Maltosyltrehalose **Trehalose** 27.4 40.2 Maltose 28.7 40.5 .alpha.-Maltosyltrehalose 21.6 19.3 .alpha.-Maltotriosyltrehalose **Trehalose** 27.4 41.1 Maltotriose 25.9 58.2 .alpha.-Maltotriosyltrehalose 19.7 0.7 .alpha.-Maltotetraosyltrehalose **Trehalose** 27.4 34.0 Maltotetraose 24.1 65.8 .alpha.-Maltotetraosyltrehalose 18.7 0.2 .alpha.-Maltopentaosyltrehalose **Trehalose** 27.4 29.1 Maltopentaose 22.6 70.6 .alpha.-Maltopentaosyltrehalose 17.8 0.3 Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

CLPR:
1. An isolated DNA molecule encoding a thermostable enzyme obtainable from a microorganism of the genus **Sulfolobus** having the following physico-chemical properties:

CLPR:
7. The isolated DNA molecule according to claim 1, which is derivable from a microorganism of the genus **Sulfolobus**.

CLPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, but not

substantially acting on maltooligosaccharides having a degree of glucose polymerization of at least 3;

USPT

US-CL-CURRENT: 426/658,435/100 ,514/54 ,514/61 ,514/777 ,514/778

US-PAT-NO: 6017899
DOCUMENT-IDENTIFIER: US 6017899 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: January 25, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
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US-CL-CURRENT: 514/53,426/658 ,435/100 ,514/54 ,514/61 ,514/777 ,514/778
ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.
14 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

ABPL:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.

BSPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses, more particularly, to a novel non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, as well as to its preparation and microorganisms capable of producing said enzyme. The present invention further relates to a composition containing a non-reducing saccharide having a **trehalose** structure as an end unit which is preparable with said enzyme, a relatively-low reducing saccharide containing said non-reducing saccharide, and/or **trehalose** prepared from these saccharides.

BSPR:
Trehalose or .alpha.,.alpha.-**trehalose** has been long known as a non-reducing saccharide consisting of glucose units. As described in Advances in Carbohydrate Chemistry, Vol.18, pp.201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), **trehalose** widely exists in microorganisms, mushrooms, insects, etc., though the content is relatively low. Since non-reducing saccharides including **trehalose** do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, non-reducing saccharides can be used with amino acids without causing browning and deterioration. Because of this, there has been in great demand to establish a method for preparation of such a non-reducing saccharide.

BSPR:
In conventional preparations of **trehalose**, as disclosed in Japanese Patent Laid-Open No.154,485/75, microorganisms are utilized, or as proposed in Japanese Patent Laid-Open No.216,695/83, maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** present in microorganisms as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, if specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, maltose as a substrate could not be used at a relatively-high concentration; (ii) Since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) it is substantially difficult to mentain the reaction systems stably and to continue their enzymatic reactions smoothly. Thus, there has not yet been realized an industrial-scale preparation of **trehalose**.

BSPR:
As regards the preparation of **trehalose**, it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No.88, pp.67-72 (August, 1992) that "In spite of a wide applicability of **trehalose**, an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been reported to be scientifically almost impossible in this field." Thus, an enzymatic preparation of **trehalose** by using starch as a material has been deemed to be scientifically very difficult.

BSPR:
In order to attain the aforementioned object, the present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme, which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates.

BSPR:
As a result, we isolated novel microorganisms of the genera Rhizobium, named as "Rhizoblum sp. M-11", and Arthrobacter, named as "Arthrobacter sp. Q36", from the respective soils in Okayama-city, Okayama, Japan, and in Soja-city, Okayama, Japan; and found that the microorganisms produce a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and that the objective non-reducing saccharides are readily prepared when the enzyme is allowed to act on reducing partial starch hydrolysates.

BSPR:
We also found that **trehalose** can be prepared by first allowing the enzyme to act on reducing partial starch hydrolysates, then subjecting the resultant non-reducing saccharides to the action of glucoamylase or .alpha.-glucosidase. Thus, the present inventors accomplished this invention. Also, we extensively screened microorganisms capable of producing the enzyme from conventional microorganisms.

BSPR:
As a result, it was found that microorganisms of the genera Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium and Terrabacter produce the present non-reducing saccharide-forming enzyme as the microorganisms of the genera Rhizobium and Arthrobacter, and we accomplished this invention. Also, we established preparations of compositions such as food products, cosmetics and pharmaceuticals which contain the present non-reducing saccharides, relatively-low reducing saccharides containing the non-reducing saccharides and/or **trehalose** prepared from these saccharides, and accomplished this invention.

DEPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses. The present invention further relates to

a microorganism capable of producing said enzyme, non-reducing saccharides prepared with said enzyme, relatively-low reducing saccharides containing said non-reducing saccharides, **trehalose** prepared from these saccharides, and compositions containing either or both of these saccharides and **trehalose**.

DEPR:
The present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and eventually found the objective microorganisms.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol.1 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Rhizobium. The microorganism is similar to those of the species Rhizobium meliloti in some properties, but they are distinguishable with the fact that the present microorganism utilizes maltose, lactose and mannitol but forms no acid, and it produces a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such a microorganism having these properties.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol.2 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Arthrobacter. The microorganism is characterized by producing a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such an enzyme.

DEPR:
The concentration of the reducing partial starch hydrolysates used as a substrate in the invention is not specifically restricted. While the present enzymatic reaction proceeds even with a 0.1% solution of a substrate, the enzymatic reaction more favorably proceeds with solutions having a concentration of 2% or higher, preferably, those having a concentration of 5-50% of a substrate, d.s.b. Under these concentrations non-reducing saccharides having a **trehalose** structure are readily formed in a satisfactorily-high yield. Suspensions containing insoluble substrates can be used in the invention. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 55.degree. C., preferably, a temperature in the range of 40-50.degree. C. The reaction pH used in the present enzymatic reaction is controlled in the range of 5-10, preferably, in the range of about 6-8. The reaction time used in the present enzymatic reaction is adequately chosen dependently on the conditions of the-enzymatic reaction.

DEPR:
If necessary, the present non-reducing saccharides having a **trehalose** structure or relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase and .alpha.-glucosidase in order to control their sweetness and reducing power or to lower their viscosity; and the resultant products can be further treated such that the remaining reducing saccharides are hydrogenated into sugar alcohols to diminish their reducing powder.

DEPR:
More particularly, **trehalose** is readily prepared by allowing glucoamylase or .alpha.-glucosidase to act on the present non-reducing saccharides or relatively-low reducing saccharides containing them. A high **trehalose** content fraction is obtained by allowing glucoamylase or .alpha.-glucosidase to act on these saccharides to form a mixture of **trehalose** and glucose, and subjecting the mixture to the aforementioned purifications such as ion-exchange column chromatography to remove glucose. The high **trehalose** content fraction can be

arbitrary purified and concentrated into a syrupy product, and, if necessary, the syrupy product can be concentrated into a supersaturated solution, followed by crystallizing hydrous- or anhydrous-crystalline **trehalose** and recovering the resultant crystal.

DEPR:
In order to prepare hydrous crystalline **trehalose,** an about 65-90% solution of **trehalose** with a purity of about 60% or higher is placed in a crystallizer, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10-90.degree. C., to obtain a massecuite containing hydrous crystalline **trehalose**. Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the invention to prepare from the massecuite hydrous crystalline **trehalose** or crystalline saccharides containing it.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline **trehalose** from the mother liquor, and, if necessary the hydrous crystalline **trehalose** is washed by spraying with a small amount of cold water to facilitate the preparation of hydrous crystalline **trehalose** with an increased purity. In the case of spray drying, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by spraying massecuites with a concentration of 70-85%, d.s.b., and a crystallinity of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto air heated to about 30-60.degree. C. In the case of block pulverization, crystalline saccharides with no hygroscopicity which are or hygroscopicity which are substantially free of hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallinity of about 10-60%, d.s.b., to stand for about 0.1-3 days in order to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

DEPR:
Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into the anhydrousform, it is generally prepared by providing a concentrated solution of **trehalose** with a moisture content less than 10%; placing the solution in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50-160.degree. C., preferably, a temperature in the range of 80-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present non-reducing saccharides are hydrolyzed by amylases such as .alpha.-amylase derived from pancreas into relatively-low molecular weight non-reducing oligosaccharides or maltooligosaccharides, and these oligosaccharides are readily hydrolyzed by .alpha.-glucosidase and intestinal enzymes into glucose and **trehalose** molecules. The resultant **trehalose** is readily hydrolyzed by trehalase into glucoses. Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** can be utilized as an energy source by the body when orally administered. These present saccharides and **trehalose** are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** have a satisfiable stability and sweetness, and those in crystalline form can be arbitrarily used as a sugar coating material for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinylpyrrolidone. These saccharides and **trehalose** have properties such as osmotic pressure-controlling ability, filler-imparting

ability, gloss-imparting ability, moisture-retaining ability, viscosity-imparting ability, substantial no fermentability, ability to prevent retrogradation of gelatinized starch, and ability to prevent crystallization of other saccharides.

DEPR:
Anhydrous crystalline **trehalose** can be arbitrarily used as a desiccant for food products, cosmetics, pharmaceuticals, and their materials and intermediates, and can be readily formed into compositions in the form of powder, granule and tablet with a satisfactory stability and quality.

DEPR:
Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient and desiccant in a variety of compositions such as food products, tobaccos, cigarettes, feeds, pet foods, cosmetics and pharmaceuticals.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, isomaltooligosaccharide, galactooligosaccharide, fructooligosaccharide, lactosucrose, sorbitol, maltitol, lactitol, dihydrocharcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as a powdery or crystalline **trehalose** prepared from these saccharides, can be used intact, or, if necessary they can be mixed with an excipient, filler and binder and formed into granules, spheres, shot-rods, plates, cubes and tablets, prior to their use.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides have the following features: (i) They have a sweetness which well harmonizes with other materials having sour-, acid-, salty-, bitter-, astringent- and delicious-tastes; and (ii) they are highly acid- and heat-resistant. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in seasonings such as amino acids, peptides, soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake), "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare" (a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), nucleic acid condiments, mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
Also, the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be freely used for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker), "arare-mochi" (a rice-cake cube), "okoshi" (a millet-and-rice cake), "mochi" (a rice paste), "manju" (a bun with a bean-jam), "uiro" (a sweet rice

jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly, pao de Castella and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickles and pickled products such as "fukujin-zuke" (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish ham, fish sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tenpura" (a Japanese deep-fat fried fish paste); "chinmi" (relish) such as "uni-no-shickara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle), "saki-surume" (dried squid strips) and "fugu-no-mirin-boshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of layer, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk products; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liquors; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix and "sokuseki-shiruco" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and beverages such as baby foods, foods for therapy, beverages supplemented with nutrition, peptide foods and frozen foods; as well as for improving the tastes and qualities of the aforementioned food-products.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be also used in feeds and pet foods for animals such as domestic animals, poultry, honey bees, silk worms and fishes in order to improve their taste preferences. These saccharides and **trehalose** can be arbitrarily used as a sweetener; taste-improving agent, quality-improving agent and stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used as a quality-improving agent and stabilizer in biologically active substances which may contain unstable effective ingredients and activities, as well as in health foods and pharmaceuticals containing the biologically active substances. Examples of such a biologically active substance are lymphokines such as .alpha.-, .beta.- and .gamma.-interferons, tumor necrosis factor-.alpha. (TNF-.alpha.), tumor necrosis factor-.beta. (TNF-.beta.), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2; hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox vaccine, tetanus toxoid, Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. By using the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides, the aforementioned biologically

active substances are arbitrarily prepared into health foods and pharmaceuticals with a satisfactorily-high stability and quality without a fear of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods for incorporating the present non-reducing saccharides, relatively-low reducing saccharides containing them and/or **trehalose** prepared from these saccharides into the above-mentioned compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. These saccharides and **trehalose** are usually incorporated into the above-mentioned compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
Fifty mg aliquots of non-reducing saccharides P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for 6 hours. The only saccharides detected in every resultant mixture on HPLC analysis were glucose and **trehalose**. The contents of the detected glucose and **trehalose**, and their molecular ratios were as shown in Table 5.

DEPR:
As evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one mole of glucose and one mole of **trehalose**; P II, hydrolyzed into two moles of glucose and one mole of **trehalose**; (iii) P III, hydrolyzed into three moles of glucose and one mole of **trehalose**; (iv) P IV, hydrolyzed into four moles of glucose and one mole of **trehalose**; and (v) P V, hydrolyzed into five moles of glucose and one mole of **trehalose**.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more moles of glucose bound to one mole of **trehalose** via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing saccharide having a degree of glucose polymerization of 3 (DP 3) and consisting of one mole of glucose bound to one mole of **trehalose**; P II, a non-reducing saccharide having DP 4 and consisting of two moles of glucose bound to one mole of **trehalose**; P III, a non-reducing saccharide having DP 5 and consisting of three moles of glucose bound to one mole of **trehalose**; P IV, a non-reducing saccharide having DP 6 and consisting of four moles of glucose bound to one mole of **trehalose**; and P V, a non-reducing saccharide having DP 7 and consisting of five moles of glucose bound to one mole of **trehalose**. It was revealed that, when .beta.-amylase was act on these non-reducing saccharides similarly as with glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one mole of maltose and one mole of P I, one mole of maltose and one mole of P II, and two moles of maltose and one mole of P I.

DEPR:
Based on these results, it was concluded that the enzymatic reaction of the present non-reducing saccharide-forming enzyme is an intramolecular reaction without changing the molecular weights of the substrates used, i.e. an intramolecular reaction without changing their degrees of glucose polymerization. It was concluded that the non-reducing saccharides P I, P II, P III, P IV and P V were the respective .alpha.-glycosyl trehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.-**trehalose** residue) of .alpha.-glucosyl **trehalose**, .alpha.-maltosyl **trehalose**, .alpha.-maltotriosyl **trehalose**, .alpha.-maltotetraosyl **trehalose** and .alpha.-maltopentaosyl **trehalose**.

DEPR:
As evident from the results in Tables 7 and 8, it was revealed that similarly as in Experiment 6 with glucoamylase the saccharide preparations P I, P II, P III, P IV and P V were hydrolyzed by .alpha.-glucosidase and rat intestinal

acetone powder into glucose and **trehalose** molecules.

DEPR:
To the resultant hydrolysate obtained with .alpha.-glucosidase or rat intestinal acetone powder was added one unit trehalase derived from pig kidney, an enzyme preparation of Sigma Chemical Company, St., Louis, USA, and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC to reveal that **trehalose,** formed from the saccharide preparations P I, P II, P III, P IV and P V, was hydrolyzed by trehalase into glucose molecules.

DEPR:
Based on these results, it was concluded that the present non-reducing saccharide-forming enzyme is a novel enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates to a non-reducing end unit, a **trehalose** residue, i.e. a **trehalose** structure.

DEPR:
By using the purified enzyme preparation obtained in Experiment 10, the preparation and the confirmation of the structure of non-reducing saccharides were conducted in accordance with the methods in Experiments 4 and 6. As a result, it was revealed that the enzyme preparation forms one or more non-reducing saccharides, which saccharide has a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher, when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
In accordance with the method in Experiment 12, non-reducing saccharides were prepared by using partially purified enzyme preparations from these known microorganisms, and their structures were studied to find that, similarly as the non-reducing saccharide-forming enzyme from Rhizoblum sp. M-11, every enzyme preparation formed non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose**; and Examples B illustrate compositions containing one or more of these saccharides and **trehalose**.

DEPR:
Forty parts by weight of "PINE-DEX #4", a partial starch hydrolysate commercialized by Matsutani Chemical Ind., Tokyo, Japan, was dissolved in 60 parts by weight of water, and the resultant solution was heated to 45.degree. C., adjusted to pH 6.5, mixed with one unit per g partial starch hydrolysate of a non-reducing saccharide-forming enzyme prepared by the method in Example A-1, and subjected to an enzymatic reaction for 96 hours while keeping at the temperature and pH. Thereafter, the reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme, diluted to give a concentration of about 20%, d.s.b., admixed with 10 units per g partial starch hydrolysate of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours, followed by heating the resultant mixture to inactivate the remaining enzyme. The mixture thus obtained was in the usual manner decolored with an activated charcoal, desalted with an ion-exchange resin, and concentrated to give a concentration of about 60%, d.s.b. The saccharide solution thus obtained contained 29.5% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 90% **trehalose,** d.s.b. The fraction was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with about 2%, d.s.b., hydrous crystalline **trehalose** as a seed crystal and gradually cooled to obtain a massecuite with a degree of crystallization of about 45%. The massecuite was

sprayed from a nozzle equipped on the top of a spraying tower at a pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air sent from the top of the spraying tower, and the resultant crystalline powder was collected on a metal wire netting conveyer provided on the basement of the spraying tower, and gradually moved out of the tower while a stream of 40.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an aging tower and aged for 10 hours to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline **trehalose**.

DEPR:
In accordance with the method in Example A-3, 30% suspension of corn starch was subjected to the action of an .alpha.-amylase specimen commercialized by Novo Industri A/S, Copenhagen, Denmark; a maltotetraose forming amylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan; and an .alpha.-amylase specimen commercialized by Ueda Chemical Co., Ltd., Osaka, Japan. The resultant mixture was autoclaved at 120.degree. C., cooled to 45.degree. C., admixed with 2 units per g starch of a non-reducing saccharide-forming enzyme prepared by the above-mentioned method, and subjected to an enzymatic reaction for 64 hours. The reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme. In accordance with the method in Example A-6, the resultant solution was subjected to the action of glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, decolored, desalted and concentrated into an about 60% solution. The saccharide solution thus obtained contained about 25% **trehalose,** d.s.b. The saccharide solution was fractionated on column chromatography using a strongly-acidic cation-exchange resin to obtain fractions rich in **trehalose**. The fractions were pooled, placed in a vessel and boiled down under a reduced pressure into a syrup with a moisture content of about 4.0%. The syrup was placed in a crystallizer and admixed with one % of anhydrous crystalline **trehalose,** as a seed crystal, with respect to the syrup, d.s.b., followed by crystallizing anhydrous crystalline **trehalose** at 95.degree. C. for 5 min while stirring. The resultant was transferred to an aluminum container and aged at 100.degree. C. for 6 hours to form a block. The resultant block was pulverized by a cutting machine and subjected to a fluidized-bed drying to obtain a powdery anhydrous crystalline **trehalose** with a moisture content of about 0.3%.

DEPR:
Three parts by weight of gum base was melted by heating until it softened, and the resultant was mixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method of Example A-6, and further mixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was kneaded by a roll in the usual manner, formed and packed to obtain the desired product.

DEPR:
Forty % "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was mixed with 2 parts by weight of a powder containing hydrous crystalline **trehalose** prepared by the method of Example A-6, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfactory taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:
Egg yolks prepared from fresh eggs were sterilized at 60-64.degree. C. by a plate heater, and the resultant liquid was mixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method of Example A-8 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was permitted to hydrate to hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting

of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method of Example A-6, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose,** one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to obtain a sugar coated tablet having a satisfiable gloss and appearance.

DEPR:
As evident from above, the present novel non-reducing saccharide-forming enzyme converts reducing partial starch hydrolysates into non-reducing saccharides in a satisfactorily-high yield under a relatively-mild enzymatic reaction condition without changing the degrees of glucose polymerization of the reducing partial starch hydrolysates. The non-reducing saccharides, which can be readily separated a nd purified, and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from es the saccharides, have a satisfactory stability, quality and mild sweetness. These products are assimilated and utilized as an energy source by the body when orally administered. These non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be arbitrarily used in compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thus, the present invention provides a novel technique to prepare in an industrial-scale and at a relatively-low cost non-reducing saccharides, which could not have been readily obtained in spite of their great demands, by using reducing partial starch hydrolysates prepared from starch as a cheap and abundant source, as well as to prepare relatively-low reducing saccharides containing the non-reducing saccharides, and **trehalose** prepared from these saccharides. The present invention has a great influence on the fields such as starch-, enzyme- and biochemical-sciences; and other industrial fields, especially, food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomable.

DEPV:
(1) The present non-reducing saccharide-forming enzyme forms non-reducing saccharides having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher without changing their degrees of glucose polymerization; and

DEPV:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is hydrolyzed by glucoamylase into one mole of **trehalose** and two moles of glucose.

DEPW:
Forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

DETL:
______ Carbon source Utilization Acid formation ______ D-Glucose + + D-Galactose + + D-Fructose + + L-Arabinose + + D-Xylose + + L-Rhamnose + + Maltose + - Sucrose + + Lactose + - **Trehalose** + - Raffinose + + Mannitol + - Dextrin + - Dulcitol + - ______

DETL:
TABLE 5 ______ Saccharide Glucose **Trehalose** Molecular ratio preparation (%) (%) (Glucose/Trehalose) ______ P I 36.2 63.8 1.07 P II 52.0 48.0 2.06 P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11

DETL:
TABLE 7 Saccharide composition of hydrolysate with .alpha.-glucosidase Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) P I 36.5 63.0 0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3 0.3

DETL:
TABLE 8 Saccharide composition of hydrolysate with rat intestinal acetone powder Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) P I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P V 73.4 26.5 0.1

CLPR:
2. The composition of claim 1, wherein the step (b) further contains a step of crystallizing said **trehalose**.

CLPR:
3. The composition of claim 2, wherein said **trehalose** is hydrous- or anhydrous-crystalline **trehalose**.

CLPR:
4. The composition of claim 1, wherein the resultant mixture in the step (b) is further subjected to column chromatography using a strongly-acidic cation-exchange resin to increase the content of **trehalose**.

CLPR:
5. The composition of claim 1, wherein the **trehalose** structure in said non-reducing saccharide is located in its end unit.

CLPR:
6. The composition of claim 5, wherein said non-reducing saccharide having a **trehalose** structure is an .alpha.-glycosyl **trehalose** shown by the formula:

CLPR:
14. A composition according to claim 1, wherein said non-reducing saccharide is an .alpha.-glycosyl **trehalose** of the formula:

CLPV:
wherein the symbol "G", "n", and "T" mean glucose, at least one integer and .alpha.,.alpha.-**trehalose** residues, respectively.

CLPV:
wherein G, N, and T mean glucose residue, at least one integer, and .alpha.,.alpha.-**trehalose** residue, respectively.

CLPW:
trehalose and non-reducing saccharides consisting of one or more glucose molecules bound to one **trehalose** molecule via the .alpha.-1,4 linkage or the .alpha.-1,6 linkage, where the total content of said non-reducing saccharides with two and three glucose molecules bound to the **trehalose** molecule is more than 43 w/w % but not more than 99 w/w %,

CLPW:
(a) contacting a solution containing reducing partial starch hydrolysates with an enzyme to form a non-reducing saccharide having a **trehalose** structure, said enzyme having the following physicochemical properties:

CLPW:
(b) contacting the product from step (a) with glucoamylase or .alpha.-glucosidase to from **trehalose**; and

CLPY:
Converting at least one reducing partial starch hydrolysate having a degree of glucose polymerization of three or more to a non-reducing saccharide having a

trehalose structure as an end unit;

ORPL:
Journal of the Chemical Society, May 1965 Letchworth, GB, pp. 3489-3490, Birch, "A method of obtaining crystalline anhydrous alphaalpha-**trehalose**".

ORPL:
Biotechnology Letters, vol. 12, No. 6, Jun. 1990, pp. 431-432; Lama et al; "Starch conversion with immobilized thermophilic Archaebacterium **Sulfolobus** Solfataricus".

ORPL:
Database WPI, Section Ch, Week 9425, Derwent Publications Ltd., London, GB; Class D16, AN 94-206435, "Alpha, alpha-**trehalose** production, comprising extraction from yeast and water, and treatment with ultrafiltration or reverse osmosis", & JP,A,06 145 186 (Kirin Brewery KK & Nippon Shokuhin Kako KK), May 24, 1994, *abstract*.

USPT

US-CL-CURRENT: 435/101,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

US-PAT-NO: 5976856
DOCUMENT-IDENTIFIER: US 5976856 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: November 2, 1999
INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/201,435/101 ,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.
1 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

ABPL:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

BSPR:
The present invention relates to a recombinant enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3.

BSPR:
Trehalose is a disaccharide which consists of 2 glucose molecules that are linked together with their reducing groups, and, naturally, it is present in fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, **trehalose** does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can advantageously sweeten food products without fear of causing unsatisfactory coloration and deterioration. **Trehalose,** however, could not have been readily prepared in a desired amount by conventional production methods, so that it has not scarcely been used for sweetening food products.

BSPR:
Conventional production methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other using a multi-enzymatic system where several enzymes are allowed to act on saccharides. The former, as disclosed in Japanese Patent Laid-Open No.154,485/75, is a method which comprises growing microorganisms such as bacteria and yeasts in nutrient culture media, and collecting **trehalose** mainly from the proliferated cells.

The latter, as disclosed in Japanese Patent Laid-Open No.216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and **trehalose**-phosphorylases to act on maltose, and recovering the formed **trehalose** from the reaction system. The former facilitates the growth of microorganisms, but has a demerit that the content in the microorganisms is at most 15 w/w %, on a dry solid basis (d.s.b.). Although the latter can readily separate **trehalose,** it is theoretically difficult to increase the **trehalose** yield by allowing such enzymes to act on substrates at a considerably-high concentration because the enzymatic reaction in itself is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

BSPR:
In view of the foregoing, the present inventors energetically screened enzymes which form non-reducing saccharides having a **trehalose** structure from amylaceous saccharides having a degree of glucose polymerization of at least 3, and have found that microorganisms such as those of the genera Rhizobium and Arthrobacter produce an absolutely novel enzyme which forms such non-reducing saccharides from such reducing amylaceous saccharides. They disclosed such an enzyme in Japanese Patent Application No.349,216/93. They also found that **trehalose** is readily formed from such non-reducing saccharides when glucoamylase or .alpha.-glucosidase acts on them.

BSPR:
It was found that the enzymes produced from the aforesaid microorganisms have an optimum temperature of about 40.degree. C., and have some difficulties in their thermostability when used to prepare **trehalose**. It is recognized in this field that the recommendable temperature in the saccharification reaction of starch or amylaceous saccharides is one which exceeds 55.degree. C. because the contamination of microorganisms will occur at a temperature of 55.degree. C. or lower, decrease the pH of the reaction mixtures, and inactivate the enzymes used. Thus, a relatively-large amount of substrates remain intact. While the use of enzymes with a poor thermostability, a great care should be taken to control the pH, and, when the pH level lowers to extremely low level, alkalis should be added to reaction mixtures to increase the pH level as quickly as possible.

BSPR:
In view of the foregoing, the present inventors screened thermostable enzyme with such a novel enzyme activity and have found that enzymes produced from microorganisms of the genus **Sulfolobus** including **Sulfolobus acidocaldarius** (ATCC 33909) are not substantially inactivated even when incubated at a temperature exceeding 55.degree. C., and they efficiently produce such non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides. These micro-organisms, however, are not sufficient in the enzyme productivity, and this requires a relatively-large scale culture to industrially produce non-reducing saccharides having a **trehalose** structure as an end unit.

BSPR:
It is an object of the present invention to provide a recombinant thermostable enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides with a degree of glucose polymerization of at least 3 by using the recombinant DNA technology.

BSPR:
It is another object of the present invention to provide a method for converting reducing amylaceous saccharides with a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit.

BSPR:
The sixth object of the present invention is attained by a method for enzymatically converting reducing amylaceous saccharides which contains a step of allowing the recombinant thermostable enzyme to act on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 to form non-reducing saccharides having a **trehalose** structure as an end unit.

BSPV:
Forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing saccharides having a degree of glucose polymerization of at least 3;

DRPR:
FIG. 1 is a figure of the optimum temperature of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 2 is a figure of the optimum pH of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 3 is a figure of the thermostability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 4 is a figure of the pH stability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The recombinant thermostable enzyme according to the present invention forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 without inactivation even when allow ed to react at a temperature exceeding 55.degree. C.

DEPR:
The present conversion method readily converts reducing amylaceous saccharide having a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit.

DEPR:
The present invention has been accomplished based on the finding of a novel enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. Such an enzyme is obtainable from cultures of microorganisms of the species **Sulfolobus acidocaldarius** (ATCC 33909). The present inventors isolated such an enzyme by using in combination a various purification methods comprising column chromatography as a main technique, studied their properties and features, and revealed that the reality is a polypeptide with the following physicochemical properties:

DEPR:
The followings are experiments which were conducted to reveal the physicochemical properties of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909):

DEPR:
Into 500-ml flasks were put 100 ml aliquots of a liquid culture medium containing 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min. After cooling the flasks a seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each liquid culture medium in each flask, followed by the incubation at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was put in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the first seed culture into the sterilized liquid culture medium in the fermenter, and culturing the microorganisms at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was placed in a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the second seed

culture into the sterilized liquid culture medium, and culturing the microorganisms at 75.degree. C. for 42 hours under an aeration condition of 100 L/min.

DEPR:
The results in Table 1 show that the purified enzyme acted on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 such as maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose to form non-reducing saccharides having a **trehalose** structure as an end unit such as .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose. In addition to these non-reducing saccharides and intact substrates, glucose and low molecular weight maltooligosaccharides as estimable hydrolysates of the substrates, were detected in the reaction mixtures, and this indicates that the purified enzyme has a hydrolytic activity. The yields of the non-reducing saccharides and hydrolysates from the substrates were respectively 30.2% and 27.6% for maltotriose, 65.4% and 18.4% for maltotetraose, and about 74-75% and about 2-3% for maltopentaose, maltohexaose and maltoheptaose. The purified enzyme formed non-reducing saccharides from maltooligosaccharides having a degree of glucose polymerization of at least 5 in a satisfactory yield, and less hydrolyzed the substrates, but did not newly form any saccharide from glucose and maltose.

DEPR:
A chromosomal DNA of **Sulfolobus acidocaldarius** (ATCC 33909) was screened by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequences in SEQ ID NOs:3 and 4, and this yielded a DNA fragment having a base sequence from the 5'-terminus consisting of about 2,200 base pairs in SEQ ID NO:2. The base sequence of the thermostable enzyme was decoded and revealing that it consists of 720 amino acids and has a partial amino acid sequence from the N-terminal in SEQ ID NO:1.

DEPR:
To 500-ml flasks were placed about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to a pH 3.0 by the addition of sulfate. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each flask, incubated at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a seed culture. About 5 L of a fresh preparation of the same liquid nutrient culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to a pH 3.0, and inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min.

DEPR:
As a control, a seed culture of Escherichia coil XLI-Blue strain or **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into a fresh preparation of the same liquid culture medium but free of ampicillin. In the case of culturing **Sulfolobus acidocaldarius** (ATCC 33909), it was cultured and treated similarly as above except that the initial pH of the nutrient culture medium and the culturing temperature were respectively set to 3.0 and 75.degree. C. Assaying the resultant enzymatic activity, one L culture of **Sulfolobus acidocaldarius** (ATCC 33909) yielded about 1.8 units of the thermostable enzyme, and the yield was significantly lower than that of transformant ST35. Escherichia coli XLI-Blue strain used as a host did not form the thermostable enzyme.

DEPR:
Thereafter, the recombinant thermostable enzyme produced by the transformant ST35 was purified similarly as in Experiments 1 and 2 and examined for properties and features and revealing that it has substantially the same physicochemical properties of the thermostable enzyme from **Sulfolobus acidocaldarius** (ATCC 33909) because (i) the recombinant thermostable enzyme has a molecular weight of about 69,000-79,000 daltons on SDS-PAGE and an isoelectric point of about 5.4-6.4 on isoelectrophoresis, and (ii) it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0)

at 85.degree. C. for 60 min. These results indicate that the present thermostable enzyme can be prepared by the recombinant DNA technology with a significantly improved yield.

DEPR:
Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of 2,200 base pairs in SEQ ID NO:5. An amino acid sequence that could be estimated from the base sequence was in SEQ ID NO:5, and it was compared with the partial amino acid sequences in SEQ ID NOs:3 and 4, and revealing that the partial amino acid sequence in SEQ ID NO:3 corresponded to that positioning from 1 to 30 in SEQ ID NO:5, and that in SEQ ID NO:4 corresponded to that positioning from 468 to 478 in SEQ ID NO:5. These results indicate that the present recombinant thermostable enzyme has the amino acid sequence from the N-terminal in SEQ ID NO:1, and, in the case of the DNA derived from **Sulfolobus acidocaldarius** (ATCC 33909), the amino acid sequence is encoded by the base sequence from the 5'-terminus in SEQ ID NO:2.

DEPR:
As is explained in the above, the thermostable enzyme, which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, was found as a result of the present inventors' long-term research. The thermostable enzyme has distinct physicochemical properties from those of other conventional enzymes. The present invention is to produce the thermostable enzyme by using the recombinant DNA technology. The present recombinant thermostable enzyme, as well as its preparation and uses, will be explained in detail with reference to the later described Examples.

DEPR:
The recombinant thermostable enzyme as referred to in the present invention means thermostable enzymes in general which are preparable by the recombinant DNA technology and capable of forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. Generally, the recombinant thermostable enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence from the N-terminal as shown in SEQ ID NO: 1, and homologous ones to it can be mentioned. Variants having amino acid sequences homologous to the one in SEQ ID NO:1 can be obtained by replacing one or more amino acids in SEQ ID NO:1 with other amino acids without substantially alternating the inherent physicochemical properties. Although even when used the same DNA and it also depends on hosts into which the DNA is introduced, the ingredients and components of nutrient culture media for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have the inherent physicochemical properties but defect one or more amino acids in SEQ ID NO:1, or those which have one or more amino acids added newly to the N-terminal after the DNA expression as the result of the modification of intracellular enzymes of the hosts. Such variants can be used in the present invention as long as they have the desired physicochemical properties.

DEPR:
The DNA usable in the present invention includes those are derived from natural resources and those which are artificially synthesized as long as they have the aforesaid base sequences. The natural resources for the DNA according to the present invention are, for example, microorganisms of the genus **Sulfolobus such as Sulfolobus acidocaldarius** (ATCC 33909), and from which genes containing the present DNA can be obtained. The aforementioned microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or .beta.-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, when treated the cells with an ultrasonic disintegrator, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or with freezing and thawing method. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment generally used in this field. To artificially synthesize the present

DNA, it can be chemically synthesized by using the base sequence in SEQ ID NO:2, or can be obtained in a plasmid form by inserting a DNA, which encodes the amino acid sequence in SEQ ID NO:1, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the objective DNA from the cells.

DEPR:
The recombinant DNA thus obtained can be introduced into appropriate host microorganisms including Escherichia coli and those of the genus Bacillus as well as actinomyces and yeasts. In the case of using Escherichia coli as a host, the DNA can be introduced thereinto by culturing the host in the presence of the recombinant DNA and calcium ion, while in the case of using a microorganism of the genus Bacillus as a host the competent cell method and the colony hybridization method can be used. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, and selecting the objective transformants which form non-reducing saccharides having a **trehalose** structure as an end unit from the reducing amylaceous saccharides.

DEPR:
The transformants thus obtained intra- and extra-cellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid culture media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with small amounts of amino acids and vitamins can be used in the invention. Examples of the carbon sources are saccharides such as unprocessed starch, starch hydrolysate, glucose, fructose, sucrose and **trehalose**. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia and salts thereof, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor, and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 20-65.degree. C. and a pH of 2-9 for about 1-6 days under aerobic conditions by the aeration-agitation method. Such cultures can be used intact as a crude enzyme, and, usually, cells in the cultures may be disrupted prior to use with ultrasonic and/or cell-wall lysis enzymes, followed by separating the thermostable enzyme from intact cells and cell debris by filtration and/or centrifugation and purifying the enzyme. The methods to purify the enzyme include conventional ones in general. From cultures intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

DEPR:
As is described above, the recombinant thermostable enzyme according to the present invention has a specific feature of forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 even when allowed to act on at a temperature exceeding 55 C. The formed non-reducing saccharides have a satisfactorily mild and high-quality sweetness as well as an adequate viscosity and moisture-retaining ability, and, as a great advantageous feature, they can sweeten food products without fear of causing unsatisfactory coloration and deterioration because they have no reducing residue within their molecules. With these features a variety of amylaceous saccharides, which have been put aside because of their reducibilities, can be converted into saccharides which have a satisfactory handleability, usefulness, and no substantial reducibility or extremely-reduced reducibility.

DEPR:
In the enzymatic conversion method according to the present invention, the present recombinant thermostable enzyme is generally allowed to coexist in an aqueous solution containing one or more of the above reducing amylaceous saccharides as a substrate, followed by the enzymatic reaction at a prescribed temperature and pH until a desired amount of the objective reducing amylaceous saccharides is formed. Although the enzymatic reaction proceeds even below a

concentration of 0.1 w/w %, d.s.b., of a substrate, a concentration of 2 w/w % or higher, d.s.b., preferably, in the range of 5-50 w/w %, d.s.b., of a substrate can be satisfactorily used when used the present conversion method in an industrial-scale production. The temperature and pH used in the enzymatic reaction are set to within the range of which does not inactivate the recombinant thermostable enzyme and allows the enzyme to effectively act on substrates, i.e. a temperature of higher than 55.degree. C. but not higher than 85.degree. C., preferably, a temperature in the range of about 56-70.degree. C., and a pH of 4-7, preferably, a pH in the range of about 5-6. The amount and reaction time suitable for the present recombinant thermostable enzyme are chosen depending on the enzymatic reaction condition. Thus, the present recombinant thermostable enzyme converts reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit, e.g. the conversion rate reaches up to about 74% when acts on maltopentaose.

DEPR:
The non-reducing saccharides thus obtained have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant. Since the non-reducing saccharides almost qualitatively form **trehalose** when received an enzymatic action of a **trehalose**-releasing enzyme as disclosed in Japanese Patent Application No.79,291/94, they can be used as an intermediate for producing **trehalose** which could not have been readily prepared.

DEPR:
The purified enzyme was assayed for properties and features by the method in Experiment 2 and revealing that it had a molecular weight of about 69,000-79,000 daltons on SDS-PAGE and a pI of about 5.4-6.4 on isoelectrophoresis, and was not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These physicochemical properties were substantially the same as those of the enzyme from a donor microorganism of **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The product had a low DE of 4.8 and contained 12.8 w/w % .alpha.-glucosyltrehalose, 11.5 w/w % .alpha.-maltosyltrehalose, 46.6 w/w % .alpha.-maltotriosyltrehalose, 2.3 w/w % .alpha.-maltotetraosyltrehalose and 3.4 w/w % .alpha.-maltopentaosyl-**trehalose,** d.s.b. Similarly as the product in Example B-1, the product has a mild and moderate sweetness and an adequate viscosity and moisture-retaining ability, and can be satisfactorily used in compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
Forty parts by weight of "PINE-DEX #4", a reducing amylaceous saccharide produced by Matsutani Chemical Ind., Co., Ltd., Kyoto, Japan, was dissolved in 60 parts by weight of water, and the solution was heated to 65.degree. C., adjusted to pH 5.5, and admixed with one unit/g reducing amylaceous saccharide, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, followed by the enzymatic reaction for 96 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, diluted up to a concentration of about 20 w/w %, d.s.b., and admixed with 10 units/g reducing amylaceous saccharide, d.s.b., of "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, followed by the enzymatic reaction for 40 hours. Thereafter, the reaction mixture was heated to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion exchanger, and concentrated into an about 60 w/w % solution. The concentrate with a **trehalose** content of 30.1 w/w %, d.s.b., was subjected to column chromatographic fractionation similarly as in Example B-2 except that "CG6000", a strong-acid cation exchange resin in Na.sup.+ -form commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used to obtain a fraction containing about 97 w/w % **trehalose,** d.s.b.

DEPR:
The fraction was concentrated up to about 75 w/w %, d.s.b., transferred to a crystallizer, and gradually cooled while stirring to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was sprayed downward from a nozzle equipped on the upper part of a spraying tower at a pressure of about 150 kg/cm.sup.2 while an about 85.degree. C. hot air was blowing downward from the upper part of the spraying tower, and the formed crystalline powder was collected on a wire-netting conveyer provided on the basement of the drying tower and gradually conveyed out of the spraying tower while an about 45.degree. C. hot air was blowing to the crystalline powder from under the conveyer. The crystalline powder thus obtained was transferred to an ageing tower and aged for 10 hours in a hot air stream to complete the crystallization and drying. Thus, a powdery hydrous crystalline **trehalose** was obtained in a yield of about 90 w/w % to the material, d.s.b.

DEPR:
As is described above, the present invention is based on the finding of a novel thermostable enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The present invention is to explore a way to produce such a thermostable enzyme in an industrial scale and in a relatively-high efficiency by the recombinant DNA technology. The present conversion method using the recombinant thermostable enzyme readily converts non-reducing amylaceous saccharides, having a degree of glucose polymerization of at least 3, into non-reducing saccharides having a **trehalose** structure as an end unit without fear of causing bacterial contamination. The non-reducing saccharides have a mild and high-quality sweetness, and, because they have no reducing residue within their molecules, they can be advantageously incorporated into compositions in general such as food products, cosmetics and pharmaceuticals without fear of causing unsatisfactory coloration and deterioration. The present recombinant thermostable enzyme is the one with a revealed amino acid sequence, so that it can be used freely in the preparations of non-reducing saccharides having a **trehalose** structure as an end unit which are premised to be used in food products and pharmaceuticals.

DEPL:
Conversion into powdery product containing crystalline **trehalose**

DEPV:
Forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing saccharides having a degree of glucose polymerization of at least 3;

CLPW:
Forming non-reducing saccharides having a **trehalose** structure as an end unit and having a degree of glucose polymerization of at least 3 from maltotetraose or reducing amylaceous saccharides having a degree of glucose polymerization of at least 3;

ORPL:
Lama, Licia et al., "Thermostable amylolytic activity from **Sulfolobus** solfataricus." Biotech Forum Europe, vol. 8, No. 4, pp. 201-203 (1991).

ORPL:
Lama, Licia et al., "Starch conversion with immobilized thermophilic archeabacterium **Sulfolobus** solfatarious.", Biotechnology Letters, vol. 12, No. 6, pp. 431-432 (Jun. 1990).

USPT

US-CL-CURRENT: 435/262,435/882 ,435/884

US-PAT-NO: 5925556
DOCUMENT-IDENTIFIER: US 5925556 A
TITLE: Method of degrading polylactic acid resin using staphylococcus hominis and staphylococcus epidermidis
DATE-ISSUED: July 20, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tokiwa; Yutaka	Tsuchiura	N/A	N/A	JPX
Jikuya; Hiroyuki	Tsukuba	N/A	N/A	JPX
Nagai; Naoko	Kameoka	N/A	N/A	JPX

US-CL-CURRENT: 435/252.1,435/262 ,435/882 ,435/884
ABSTRACT:

A microorganism belonging to the genus Staphylococcus or the genus Streptomyces which is capable of degrading a polylactic acid resin. A method of degrading a polylactic acid resin including a step of culturing a microorganism capable of degrading a polylactic acid resin in a medium containing a polylactic acid resin. In particular, the microorganisms Staphylococcus hominis FERM BP-6108 and Staphylococcus epidermidis FERM BP-6109.

11 Claims, 7 Drawing figures
Exemplary Claim Number: 3
Number of Drawing Sheets: 5

DETL:
TABLE 1-3 ______ Genera of Bacteria
______ Cardiobacterium Thiobacillus
Streptobacillus **Sulfolobus** Calymmatobacterium Thiobacterium BACTEROIDACEAE Macromonas Bacteroides Thiovulum Fusobacterium Thiospira Leptotrichia SIDEROCAPSACEAE Desulfovibrio Siderocapsa Butyrivibrio Naumaniella Succinivibrio Ochrobium Succinimonas Siderococcus Lachnospira METHANOBACTERIACEAE Selenomonas Methanobacterium NEISSERIACEAE Methanosarcina Neisseria Methanococcus Branbamella MICROCOCCACEAE Moroxella Micrococcus Acinetobacter Staphylococcus Paracoccus Planococcus Lampropedia STREPTOCOCCACEAE VEILLONELLACEAE Streptococcus Veillonella Leuconostoc Acidaminococcus Pediococcus Megasphaera Aerococcus NITROBACTERACEAE Gemella Nitrobacter PERPTOCOCCACEAE Nitrospina Peptococcus Nitrococcus Peptostreptoccus Nitrosomonas Ruminococcus Nitrosospira Sarcina Nitrosococcus Nitrosolobus ______

DETL:
TABLE 3 ______ Staphylococcus (FERM BP-6108)
Strain Spherule, cream color, Colony Morphology 1 mm in diameter
______ Gram Staining + Spore Formation -
Motility - Aerial Mycelium - Growth at: 30.degree. C. + 37.degree. C. + 50.degree. C. - Catalase + Oxidase - OF Test + Glucose + Maltose + Mannose + Raffinose - **Trehalose** + Fructose + Xylose - Sucrose + Mannitol + Lactose - Melibiose - ______

DETL:
TABLE 4 ______ Staphylococcus (FERM BP-6109)
Strain Spherule, white color, Colony Morphology 1 mm in diameter
______ Gram Staining + Spore Formation -
Motility - Aerial Mycelium - Growth at: 30.degree. C. - 37.degree. C. - 50.degree. C. + Catalase + Oxidase - OF Test + Glucose + Maltose + Mannose + Raffinose - **Trehalose** - Fructose + Xylose - Sucrose + Mannitol - Lactose + Melibiose - ______

USPT

US-CL-CURRENT: 435/101,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

US-PAT-NO: 5922578
DOCUMENT-IDENTIFIER: US 5922578 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: July 13, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/101 ,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

ABSTRACT:

Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

7 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

ABPL:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

BSPR:
The present invention relates to a recombinant enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3.

BSPR:
Trehalose is a disaccharide which consists of 2 glucose molecules that are linked together with their reducing groups, and, naturally, it is present in fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, **trehalose** does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can advantageously sweeten food products without fear of causing unsatisfactory coloration and deterioration. **Trehalose,** however, could not have been readily prepared in a desired amount by conventional production methods, so that it has not scarcely been used for sweetening food products.

BSPR:
Conventional production methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other using a multi-enzymatic system where several enzymes are allowed to act on saccharides. The former, as disclosed in Japanese Patent Laid-Open No. 154,485/75, is a method which comprises growing microorganisms such as bacteria and yeasts in nutrient culture media, and collecting **trehalose** mainly from the proliferated cells.

The latter, as disclosed in Japanese Patent Laid-Open No. 216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and **trehalose**-phosphorylases to act on maltose, and recovering the formed **trehalose** from the reaction system. The former facilitates the growth of microorganisms, but has a demerit that the content in the microorganisms is at most 15 w/w %, on a dry solid basis (d.s.b.). Although the latter can readily separate **trehalose,** it is theoretically difficult to increase the **trehalose** yield by allowing such enzymes to act on substrates at a considerably-high concentration because the enzymatic reaction in itself is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

BSPR:
In view of the foregoing, the present inventors energetically screened enzymes which form non-reducing saccharides having a **trehalose** structure from amylaceous saccharides having a degree of glucose polymerization of at least 3, and have found that microorganisms such as those of the genera Rhizobium and Arthrobacter produce an absolutely novel enzyme which forms such non-reducing saccharides from such reducing amylaceous saccharides. They disclosed such an enzyme in Japanese Patent Application No. 349,216/93. They also found that **trehalose** is readily formed from such non-reducing saccharides when glucoamylase or .alpha.-glucosidase acts on them.

BSPR:
It was found that the enzymes produced from the aforesaid microorganisms have an optimum temperature of about 40.degree. C., and have some difficulties in their thermostability when used to prepare **trehalose**. It is recognized in this field that the recommendable temperature in the saccharification reaction of starch or amylaceous saccharides is one which exceeds 55.degree. C. because the contamination of microorganisms will occur at a temperature of 55.degree. C. or lower, decrease the pH of the reaction mixtures, and inactivate the enzymes used. Thus, a relatively-large amount of substrates remain intact. While the use of enzymes with a poor thermostability, a great care should be taken to control the pH, and, when the pH level lowers to extremely low level, alkalis should be added to reaction mixtures to increase the pH level as quickly as possible.

BSPR:
In view of the foregoing, the present inventors screened thermostable enzyme with such a novel enzyme activity and have found that enzymes produced from microorganisms of the genus **Sulfolobus** including **Sulfolobus acidocaldarius** (ATCC 33909) are not substantially inactivated even when incubated at a temperature exceeding 55.degree. C., and they efficiently produce such non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides. These micro-organisms, however, are not sufficient in the enzyme productivity, and this requires a relatively-large scale culture to industrially produce non-reducing saccharides having a **trehalose** structure as an end unit.

BSPR:
It is an object of the present invention to provide a recombinant thermostable enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides with a degree of glucose polymerization of at least 3 by using the recombinant DNA technology.

BSPR:
It is another object of the present invention to provide a method for converting reducing amylaceous saccharides with a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit.

BSPR:
The sixth object of the present invention is attained by a method for enzymatically converting reducing amylaceous saccharides which contains a step of allowing the recombinant thermostable enzyme to act on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 to form non-reducing saccharides having a **trehalose** structure as an end unit.

BSPW:
Forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing saccharides having a degree of glucose polymerization of at least 3;

DRPR:
FIG. 1 is a figure of the optimum temperature of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 2 is a figure of the optimum pH of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 3 is a figure of the thermostability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 4 is a figure of the pH stability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The recombinant thermostable enzyme according to the present invention forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 without inactivation even when allowed to react at a temperature exceeding 55.degree. C.

DEPR:
The present conversion method readily converts reducing amylaceous saccharide having a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit.

DEPR:
The present invention has been accomplished based on the finding of a novel enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. Such an enzyme is obtainable from cultures of microorganisms of the species **Sulfolobus acidocaldarius** (ATCC 33909). The present inventors isolated such an enzyme by using in combination a various purification methods comprising column chromatography as a main technique, studied their properties and features, and revealed that the reality is a polypeptide with the following physicochemical properties:

DEPR:
The followings are experiments which were conducted to reveal the physicochemical properties of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909):

DEPR:
Into 500-ml flasks were put 100 ml aliquots of a liquid culture medium containing 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min. After cooling the flasks a seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each liquid culture medium in each flask, followed by the incubation at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was put in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the first seed culture into the sterilized liquid culture medium in the fermenter, and culturing the microorganisms at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was placed in a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the second seed

culture into the sterilized liquid culture medium, and culturing the microorganisms at 75.degree. C. for 42 hours under an aeration condition of 100 L/min.

DEPR:
The results in Table 1 show that the purified enzyme acted on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 such as maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose to form non-reducing saccharides having a **trehalose** structure as an end unit such as .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose. In addition to these non-reducing saccharides and intact substrates, glucose and low molecular weight maltooligosaccharides as estimable hydrolysates of the substrates, were detected in the reaction mixtures, and this indicates that the purified enzyme has a hydrolytic activity. The yields of the non-reducing saccharides and hydrolysates from the substrates were respectively 30.2% and 27.6% for maltotriose, 65.4% and 18.4% for maltotetraose, and about 74-75% and about 2-3% for maltopentaose, maltohexaose and maltoheptaose. The purified enzyme formed non-reducing saccharides from maltooligosaccharides having a degree of glucose polymerization of at least 5 in a satisfactory yield, and less hydrolyzed the substrates, but did not newly form any saccharide from glucose and maltose.

DEPR:
A chromosomal DNA of **Sulfolobus acidocaldarius** (ATCC 33909) was screened by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequences in SEQ ID NOs:3 and 4, and this yielded a DNA fragment having a base sequence from the 5'-terminus consisting of about 2,200 base pairs in SEQ ID NO:2. The base sequence of the thermostable enzyme was decoded and revealing that it consists of 720 amino acids and has a partial amino acid sequence from the N-terminal in SEQ ID NO:1.

DEPR:
To 500-ml flasks were placed about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to a pH 3.0 by the addition of sulfate. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each flask, incubated at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a seed culture. About 5 L of a fresh preparation of the same liquid nutrient culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to a pH 3.0, and inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min.

DEPR:
As a control, a seed culture of Escherichia coli XLI-Blue strain or **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into a fresh preparation of the same liquid culture medium but free of ampicillin. In the case of culturing **Sulfolobus acidocaldarius** (ATCC 33909), it was cultured and treated similarly as above except that the initial pH of the nutrient culture medium and the culturing temperature were respectively set to 3.0 and 75.degree. C. Assaying the resultant enzymatic activity, one L culture of **Sulfolobus acidocaldarius** (ATCC 33909) yielded about 1.8 units of the thermostable enzyme, and the yield was significantly lower than that of transformant ST35. Escherichia coli XLI-Blue strain used as a host did not form the thermostable enzyme.

DEPR:
Thereafter, the recombinant thermostable enzyme produced by the transformant ST35 was purified similarly as in Experiments 1 and 2 and examined for properties and features and revealing that it has substantially the same physicochemical properties of the thermostable enzyme from **Sulfolobus acidocaldarius** (ATCC 33909) because (i) the recombinant thermostable enzyme has a molecular weight of about 69,000-79,000 daltons on SDS-PAGE and an isoelectric point of about 5.4-6.4 on isoelectrophoresis, and (ii) it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0)

at 85.degree. C. for 60 min. These results indicate that the present thermostable enzyme can be prepared by the recombinant DNA technology with a significantly improved yield.

DEPR:
Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of 2,200 base pairs in SEQ ID NO:5. An amino acid sequence that could be estimated from the base sequence was in SEQ ID NO:5, and it was compared with the partial amino acid sequences in SEQ ID NOs:3 and 4, and revealing that the partial amino acid sequence in SEQ ID NO:3 corresponded to that positioning from 1 to 30 in SEQ ID NO:5, and that in SEQ ID NO:4 corresponded to that positioning from 468 to 478 in SEQ ID NO:5. These results indicate that the present recombinant thermostable enzyme has the amino acid sequence from the N-terminal in SEQ ID NO:1, and, in the case of the DNA derived from **Sulfolobus acidocaldarius** (ATCC 33909), the amino acid sequence is encoded by the base sequence from the 5'-terminus in SEQ ID NO:2.

DEPR:
As is explained in the above, the thermostable enzyme, which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, was found as a result of the present inventors' long-term research. The thermostable enzyme has distinct physicochemical properties from those of other conventional enzymes. The present invention is to produce the thermostable enzyme by using the recombinant DNA technology. The present recombinant thermostable enzyme, as well as its preparation and uses, will be explained in detail with reference to the later described Examples.

DEPR:
The recombinant thermostable enzyme as referred to in the present invention means thermostable enzymes in general which are preparable by the recombinant DNA technology and capable of forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. Generally, the recombinant thermostable enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence from the N-terminal as shown in SEQ ID NO:1, and homologous ones to it can be mentioned. Variants having amino acid sequences homologous to the one in SEQ ID NO:1 can be obtained by replacing one or more bases in SEQ ID NO:1 with other bases without substantially alternating the inherent physicochemical properties. Although even when used the same DNA and it also depends on hosts into which the DNA is introduced, the ingredients and components of nutrient culture media for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have the inherent physicochemical properties but defect one or more amino acids in SEQ ID NO:1, or those which have one or more amino acids added newly to the N-terminal after the DNA expression as the result of the modification of intracellular enzymes of the hosts. Such variants can be used in the present invention as long as they have the desired physicochemical properties.

DEPR:
The DNA usable in the present invention includes those are derived from natural resources and those which are artificially synthesized as long as they have the aforesaid base sequences. The natural resources for the DNA according to the present invention are, for example, microorganisms of the genus **Sulfolobus such as Sulfolobus acidocaldarius** (ATCC 33909), and from which genes containing the present DNA can be obtained. The aforementioned microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or .beta.-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, when treated the cells with an ultrasonic disintegrator, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or with freezing and thawing method. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment generally used in this field. To artificially synthesize the present

DNA, it can be chemically synthesized by using the base sequence in SEQ ID NO:2, or can be obtained in a plasmid form by inserting a DNA, which encodes the amino acid sequence in SEQ ID NO:1, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the objective DNA from the cells.

DEPR:
The recombinant DNA thus obtained can be introduced into appropriate host microorganisms including Escherichia coli and those of the genus Bacillus as well as actinomyces and yeasts. In the case of using Escherichia coli as a host, the DNA can be introduced thereinto by culturing the host in the presence of the recombinant DNA and calcium ion, while in the case of using a microorganism of the genus Bacillus as a host the competent cell method and the colony hybridization method can be used. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, and selecting the objective transformants which form non-reducing saccharides having a trehalose structure as an end unit from the reducing amylaceous saccharides.

DEPR:
The transformants thus obtained intra- and extra-cellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid culture media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with small amounts of amino acids and vitamins can be used in the invention. Examples of the carbon sources are saccharides such as unprocessed starch, starch hydrolysate, glucose, fructose, sucrose and trehalose. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia and salts thereof, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor, and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 20-65.degree. C. and a pH of 2-9 for about 1-6 days under aerobic conditions by the aeration-agitation method. Such cultures can be used intact as a crude enzyme, and, usually, cells in the cultures may be disrupted prior to use with ultrasonic and/or cell-wall lysis enzymes, followed by separating the thermostable enzyme from intact cells and cell debris by filtration and/or centrifugation and purifying the enzyme. The methods to purify the enzyme include conventional ones in general. From cultures intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

DEPR:
As is described above, the recombinant thermostable enzyme according to the present invention has a specific feature of forming non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 even when allowed to act on at a temperature exceeding 55.degree. C. The formed non-reducing saccharides have a satisfactorily mild and high-quality sweetness as well as an adequate viscosity and moisture-retaining ability, and, as a great advantageous feature, they can sweeten food products without fear of causing unsatisfactory coloration and deterioration because they have no reducing residue within their molecules. With these features a variety of amylaceous saccharides, which have been put aside because of their reducibilities, can be converted into saccharides which have a satisfactory handleability, usefulness, and no substantial reducibility or extremely-reduced reducibility.

DEPR:
In the enzymatic conversion method according to the present invention, the present recombinant thermostable enzyme is generally allowed to coexist in an aqueous solution containing one or more of the above reducing amylaceous saccharides as a substrate, followed by the enzymatic reaction at a prescribed temperature and pH until a desired amount of the objective reducing amylaceous

saccharides is formed. Although the enzymatic reaction proceeds even below a concentration of 0.1 w/w %, d.s.b., of a substrate, a concentration of 2 w/w % or higher, d.s.b., preferably, in the range of 5-50 w/w %, d.s.b., of a substrate can be satisfactorily used when used the present conversion method in an industrial-scale production. The temperature and pH used in the enzymatic reaction are set to within the range of which does not inactivate the recombinant thermostable enzyme and allows the enzyme to effectively act on substrates, i.e. a temperature of higher than 55.degree. C. but not higher than 85.degree. C., preferably, a temperature in the range of about 56-70.degree. C., and a pH of 4-7, preferably, a pH in the range of about 5-6. The amount and reaction time suitable for the present recombinant thermostable enzyme are chosen depending on the enzymatic reaction condition. Thus, the present recombinant thermostable enzyme converts reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 into non-reducing saccharides having a **trehalose** structure as an end unit, e.g. the conversion rate reaches up to about 74% when acts on maltopentaose.

DEPR:
The non-reducing saccharides thus obtained have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant. Since the non-reducing saccharides almost qualitatively form **trehalose** when received an enzymatic action of a **trehalose**-releasing enzyme as disclosed in Japanese Patent Application No. 79,291/94, they can be used as an intermediate for producing **trehalose** which could not have been readily prepared.

DEPR:
The purified enzyme was assayed for properties and features by the method in Experiment 2 and revealing that it had a molecular weight of about 69,000-79,000 daltons on SDS-PAGE and a pI of about 5.4-6.4 on isoelectrophoresis, and was not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These physicochemical properties were substantially the same as those of the enzyme from a donor microorganism of **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The product had a low DE of 4.8 and contained 12.8 w/w % .alpha.-glucosyltrehalose, 11.5 w/w % .alpha.-maltosyltrehalose, 46.6 w/w % .alpha.-maltotriosyltrehalose, 2.3 w/w % .alpha.-maltotetraosyltrehalose and 3.4 w/w % .alpha.-maltopentaosyl-**trehalose,** d.s.b. Similarly as the product in Example B-1, the product has a mild and moderate sweetness and an adequate viscosity and moisture-retaining ability, and can be satisfactorily used in compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
Forty parts by weight of "PINE-DEX #4", a reducing amylaceous saccharide produced by Matsutani Chemical Ind., Co., Ltd., Kyoto, Japan, was dissolved in 60 parts by weight of water, and the solution was heated to 65.degree. C., adjusted to pH 5.5, and admixed with one unit/g reducing amylaceous saccharide, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, followed by the enzymatic reaction for 96 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, diluted up to a concentration of about 20 w/w %, d.s.b., and admixed with 10 units/g reducing amylaceous saccharide, d.s.b., of "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, followed by the enzymatic reaction for 40 hours. Thereafter, the reaction mixture was heated to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion exchanger, and concentrated into an about 60 w/w % solution. The concentrate with a **trehalose** content of 30.1 w/w %, d.s.b., was subjected to column chromatographic fractionation similarly as in Example B-2 except that "CG6000", a strong-acid cation exchange resin in Na.sup.+ -form commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used to obtain a fraction containing about 97 w/w % **trehalose,** d.s.b.

DEPR:
The fraction was concentrated up to about 75 w/w %, d.s.b., transferred to a crystallizer, and gradually cooled while stirring to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was sprayed downward from a nozzle equipped on the upper part of a spraying tower at a pressure of about 150 kg/cm.sup.2 while an about 85.degree. C. hot air was blowing downward from the upper part of the spraying tower, and the formed crystalline powder was collected on a wire-netting conveyer provided on the basement of the drying tower and gradually conveyed out of the spraying tower while an about 45.degree. C. hot air was blowing to the crystalline powder from under the conveyer. The crystalline powder thus obtained was transferred to an ageing tower and aged for 10 hours in a hot air stream to complete the crystallization and drying. Thus, a powdery hydrous crystalline **trehalose** was obtained in a yield of about 90 w/w % to the material, d.s.b.

DEPR:
As is described above, the present invention is based on the finding of a novel thermostable enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The present invention is to explore a way to produce such a thermostable enzyme in an industrial scale and in a relatively-high efficiency by the recombinant DNA technology. The present conversion method using the recombinant thermostable enzyme readily converts non-reducing amylaceous saccharides, having a degree of glucose polymerization of at least 3, into non-reducing saccharides having a **trehalose** structure as an end unit without fear of causing bacterial contamination. The non-reducing saccharides have a mild and high-quality sweetness, and, because they have no reducing residue within their molecules, they can be advantageously incorporated into compositions in general such as food products, cosmetics and pharmaceuticals without fear of causing unsatisfactory coloration and deterioration. The present recombinant thermostable enzyme is the one with a revealed amino acid sequence, so that it can be used freely in the preparations of non-reducing saccharides having a **trehalose** structure as an end unit which are premised to be used in food products and pharmaceuticals.

DEPC:
Conversion into Powdery Product Containing Crystalline **Trehalose**

DEPW:
Forming non-reducing saccharides having a **trehalose** structure as an end unit from reducing saccharides having a degree of glucose polymerization of at least 3;

CLPR:
1. A method for converting a reducing amylaceous saccharide to form a non-reducing saccharide having a **trehalose** structure as an end unit, comprising the steps of:

CLPR:
5. The method according to claim 1, wherein said non-reducing saccharide is selected from the group consisting of .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyl**-trehalose,** .alpha.-maltopentaosyltrehalose, and mixtures thereof.

CLPR:
6. A method for converting a reducing amylaceous saccharide to form a non-reducing saccharide having a **trehalose** structure as an end unit, comprising the steps of:

CLPV:
transforming a host microorganism with a recombinant DNA molecule encoding a thermostable enzyme, which forms a non-reducing saccharide having a **trehalose** structure as an end unit from a reducing amylaceous saccharide having a decree of glucose polymerization of 3 or higher, to obtain a recombinant microorganism, wherein the thermostable enzyme has an amino acid sequence selected from the group consisting of SEQ ID NO:1 and functional variants

thereof in which one amino acid residue in SEQ ID NO:1 is replaced with a different amino acid or deleted, or one or more amino acids are added to the N-terminus of SEQ ID NO:1, without substantially losing the physicochemical properties of said enzyme, which physicochemical properties include the following:

CLPV:
subjecting a reducing amylaceous saccharide having a degree of glucose polymerization of at least 3 to the action of the recovered enzyme to convert the reducing amylaceous saccharide and form a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

CLPV:
transforming a host cell with a recombinant DNA molecule encoding a thermostable enzyme, which forms a non-reducing saccharide having a **trehalose** structure as an end unit from a reducing amylaceous saccharide having a degree of glucose polymerization of 3 or higher, to obtain a recombinant microorganism, wherein the thermostable enzyme has the following physicochemical properties:

CLPV:
subjecting a reducing amylaceous saccharide having a degree of glucose polymerization of at least 3 to the action of the recovered enzyme to convert the reducing amylaceous saccharide and form a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

CLPX:
Forming non-reducing saccharides, having a **trehalose** structure as an end unit and a degree of polymerization of at least 3 from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3;

CLPX:
Forming non-reducing saccharides, having a **trehalose** structure as an end unit and a degree of polymerization of at least 3 from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3;

ORPL:
Lama, Licia et al., "Thermostable amylolytic activity from **sulfolobus** solfataricus." Biotech from Europe, vol. 8, No. 4, pp. 201-203 (1991).

ORPL:
Lama, Licia et al., "Starch conversion with immobilized thermophilic archaebacterium **sulfolobus** solfataricus." Biotechnology Letters, vol. 12, No. 6, pp. 431-432 (Jun. 1990).

USPT

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TITLE: Non-reducing saccharide and its production and use
DATE-ISSUED: July 6, 1999
INVENTOR-INFORMATION:

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ABSTRACT:
In the production of non-reducing saccharides such as **trehalose,** alpha-glycosyl trehaloses and alpha-glycosyl alpha-glycosides where a solution of liquefied starch is subjected either to non-reducing saccharide-forming enzyme or non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme, combinations with starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase improve the yields for such non-reducing saccharides to levels which are hardly attainable only with reducing-saccharide-forming enzyme and **trehalose**-releasing enzyme. The non-reducing saccharides and less reducing reducing saccharides containing the same commonly bear a variety of desirable properties which make them useful in a variety of compositions including food products, cosmetics and medicines.
22 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

ABPL:
In the production of non-reducing saccharides such as **trehalose,** alpha-glycosyl trehaloses and alpha-glycosyl alpha-glycosides where a solution of liquefied starch is subjected either to non-reducing saccharide-forming enzyme or non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme, combinations with starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase improve the yields for such non-reducing saccharides to levels which are hardly attainable only with reducing-saccharide-forming enzyme and **trehalose**-releasing enzyme. The non-reducing saccharides and less reducing reducing saccharides containing the same commonly bear a variety of desirable properties which make them useful in a variety of compositions including food products, cosmetics and medicines.

BSPR:
The present invention relates to non-reducing saccharides and their production and use, in particular, to non-reducing saccharides including **trehalose** and non-reducing saccharides bearing at their ends or within their molecules **trehalose** structures, a process to produce the same from starch and a composition which contains such a non-reducing saccharide or less reducing saccharide containing the same.

BSPR:
Trehalose (alpha,alpha-**trehalose**) has been known from ancient times as a non-reducing saccharide composed of glucose, and as described in Advances in Carbohydrate Chemistry, published by Academic Press Inc., New York, N.Y., USA, Vol.18, pp.201-225 (1963) and Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), its trace but extensive distribution is found in microorganisms, mushrooms and insects. Since non-reducing saccharides cause no aminocarbonyl reactions with substances bearing amino groups such as amino acids and proteins and therefore neither deteriorate nor alter them, as is the case with **trehalose** the saccharides have been deemed to be useful in utilizing and processing such substances with no fears of their browning and

deterioration: Thus establishment of processes which would enable their industrial-scale production has been logd desired.

BSPR:
There have been known several processes to produce **trehalose,** for example, those using microorganism cells as disclosed in Japanese Patent Kokai No. 154,485/75 and those converting maltose by combination of maltose phosphorylase and **trehalose** phosphorylase. The former process using micro-organism cells is however inadequate for industrial-scale process because the **trehalose** content in microorganism cells as starting material is generally low, i.e. less than 15 w/w % (the percentages appeared hereinafter mean "w/w %" unless specified otherwise), and the extraction and purification steps for **trehalose** are very complicated. The latter process using maltose phosphorylase and **trehalose** phosphorylase has not been realized in industrial scale due to the demerits that both enzymes commonly act via glucose-1-phosphate and this hinders elevated concentrations for substrates. The yield for **trehalose** is low because both enzymes irreversibly act in the same reaction system, and further that such reaction system is very difficult to maintain stable and proceed smoothly.

BSPR:
In connection with this, Gekkan Food Chemical (Monthly Food Chemical), "Recent Aspects and Issues in Utilization and Development of Starch", August, pp.67-72 (1992) comments in the corner of "Oligosaccharides" that although **trehalose** would have very extensive uses, its enzymatic production using any direct saccharide-transferring or hydrolyzing reactions has been deemed to be scientifically impossible at the present time, confirming that the production of **trehalose** from starch as material using enzymatic reactions has been deemed to be scientifically impossible.

BSPR:
To solve these, the present inventors disclose in Japanese Patent Application No. 349,216/93 a novel non-reducing saccharide-forming enzyme (referred to as "non-reducing saccharide-forming enzyme" hereinafter) which is capable of forming non-reducing saccharides bearing at their ends **trehalose** structures from one or more reducing partial starch hydrolysates with glucose polymerization degrees of 3 or higher, thus establishing non-reducing saccharides bearing at their molecular ends **trehalose** structures and less reducing saccharides containing the same, as well as establishing a process to produce **trehalose** from these saccharides using the non-reducing saccharide-forming enzyme.

BSPR:
It was however found later that the non-reducing saccharides obtained by this process were less in reducing power but somewhat too high in viscosity when reducing partial starch hydrolysates used as starting material were relatively large molecules, while one obtained an insufficient decrease of reducing power when reducing partial starch hydrolysates used as starting material were relatively small molecules. Also was found that production of **trehalose** where the non-reducing saccharides thus obtained were subjected to glucoamylase was too low in the yield from starch as material to enable industrial-scale production of **trehalose**. To improve these, there has been in a great demand to establish any methods which would give much smaller non-reducing saccharides from reducing partial starch hydrolysates at higher yields.

BSPR:
The present inventors also disclose in Japanese Patent Application No. 79,291/94 a novel **trehalose**-releasing enzyme (referred to as "**trehalose**-releasing enzyme" hereinafter) which specifically hydrolyzes the linkages between the **trehalose** moieties and other moieties in non-reducing saccharides with glucose polymerization degrees of 3 or higher, as well as establishing a process to produce **trehalose** at a relatively high yield where the non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme are used in combination. To produce **trehalose** in industrial scale, there has been however in a great expectation to establish any processes which would realize an improved yield for **trehalose**.

BSPR:
The present invention provides a process to produce from starch as low cost and

consistently available material non-reducing saccharides and less reducing saccharides containing the same including relatively small non-reducing saccharides bearing at their ends **trehalose** structures (referred to as "alpha-glycosyl **trehalose**" hereinafter), non-reducing saccharides bearing at both ends in their molecule **trehalose** structures, in other words, those bearing within their molecules **trehalose** structures (referred to as "alpha-glycosyl alpha-glycoside" hereinafter) and **trehalose** at elevated yields, as well as to provide their use.

BSPR:
To solve these objects, the present inventors have energetically investigated various processes to produce non-reducing saccharides using starch as starting material. As the result, the present inventors found that the objects were attained by the process where starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase is used in combination when a solution of liquefied starch is subjected either to non-reducing saccharide-forming enzyme or to non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme. Thus the present inventors accomplished the present invention.

BSPR:
More particularly, it was found that in the production of alpha-glycosyl trehaloses or less reducing saccharides containing the same where a solution of liquefied starch with a relatively low DE, desirably, DE lower than 15, is subjected to non-reducing saccharide-forming enzyme, less reducing saccharides containing non-reducing saccharides obtained by subjecting further to starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase became lower in molecular weight and viscosity and more easily handleable with no substantial increases in reducing power than in case of subjecting saccharides only to non-reducing saccharide-forming enzyme. Also was found that after subjecting the less reducing saccharides to glucoamylase, the **trehalose** contents in their structures were extensively elevated. Further it was found that in the production of **trehalose** where a solution of liquefied starch with a relatively low DE, desirably, DE lower than 15, was subjected to non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme, the yield for **trehalose** was much more improved by subjecting the startch further to starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase than in case of subjecting the startch only to non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme. The non-reducing saccharides and less reducing saccharide containing the same thus obtained are high in stability, easily handleable and therefore feasible in extended uses, for example, in a variety of compositions including food products, cosmetics and medicines.

DRPR:
FIG. 9 shows the elution patterns on DEAE TOYOPEARL for the **trehalose**-releasing enzyme and non-reducing saccharide-forming enzyme both according to the present invention.

DRPR:
FIG. 10 shows the effect of temperature on the activity of the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DRPR:
FIG. 11 shows the effect of pH on the activity of the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DRPR:
FIG. 12 shows the thermal stability of the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DRPR:
FIG. 13 shows the pH stability of the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DRPR:
FIG. 14 shows the effect of temperature on the activity of the **trehalose**-releasing enzyme derived from Arthrobacter species Q36.

DRPR:

FIG. 15 shows the effect of pH on the activity of the **trehalose**-releasing enzyme derived from Arthrobacter species Q36.

DRPR:
FIG. 16 shows the thermal stability of the **trehalose**-releasing enzyme derived from Arthrobacter species Q36.

DRPR:
FIG. 17 shows the pH stability of the **trehalose**-releasing enzyme derived from Arthrobacter species Q36.

DEPR:
First, the non-reducing saccharide-forming enzymes feasible in the present invention are those which are capable of forming alpha-glycosyl trehaloses from one or more reducing partial starch hydrolysates with glucose polymerization degrees of 3 or higher in solutions of starch which has been liquefied to a relatively low DE: Examples of such an enzyme are those derived from micro-organisms of the genera Rhizobium, Arthrobacter, Brevibacterium, Flabobacterium, Micrococcus, Curtobacterium, Mycobacterium and Terrabacter which are disclosed in Japan Patent Application No. 349,216/93. If necessary, heat-resistant non-reducing saccharide-forming enzymes, for example, those from the genus **Sulfolobus** as disclosed in Japanese Patent Application No. 166,011/94 by the same applicant, can be arbitrarily used. The **trehalose**-releasing enzymes are such as those which specifically hydrolyze the linkages between the **trehalose** moieties and the other moieties in alpha-glycosyl trehaloses which have been formed by subjecting a solution of liquefied starch to non-reducing saccharide-forming enzyme. Examples of such an enzyme are those derived from the genera Rhizobium, Arthrobacter, Brevibacterium and Micrococcus which are all disclosed in Japanese Patent Application No. 79,291/94. If necessary, heat-resistant **trehalose**-releasing enzymes, for example, those as disclosed in Japanese Patent Application No. 166,126/94 by the same applicant, can be arbitrarily used. To prepare non-reducing saccharide-forming enzyme and/or **trehalose**-releasing enzyme, micro-organisms capable of producing either of both of the enzymes are cultivated.

DEPR:
Such cultivation is carried out on synthetic or natural culture media where the objective micro-organism can grow and produce non-reducing saccharide-forming enzyme and/or **trehalose**-releasing enzyme. The carbon sources are substances which are assimilable by such a micro-organism including saccharides, for example, glucose, fructose, lactose, sucrose, mannitol, sorbitol, sugar syrup and reducing partial starch hydrolysates, and organic acids and their salts, for example, citric acid, succinic acid and their salts. The concentration of carbon source in culture media is arbitrarily chosen depending on the types of particular carbon sources. For example, in the case of reducing partial starch hydrolysates, preferable concentrations are usually 20% or lower, preferably, 5% or lower with viewpoints of growth and proliferation of micro-organisms. Examples of nitrogen sources are inorganic salts such as ammonium salts and nitrates and organic nitrogen compounds such as urea, corn steep liquor, casein, peptone, yeast extract and meat extract. Example of minerals are calcium salts, magnesium salts, potassium salts, sodium salts, phosphates, manganese salts, zinc salts, iron salts, copper salts, molybdenum salts and cobalt salts. If necessary, amino acids and vitamins can be arbitrarily used.

DEPR:
While the **trehalose**-releasing enzymes obtained as above generally have the following physicochemical properties:

DEPR:
Trehalose-releasing enzyme is assayed as follows: 4 ml of 1.25 w/v % maltotriosyl **trehalose** or alpha-maltotetraosyl alpha-D-glucoside as substrate in 50 mM phosphate buffer (pH7.0) is added with 1 ml of enzyme liquid, allowed to react at 40.degree. C. for 30 minutes, added with Somogyi's copper liquid to suspend the reaction and assayed for reducing power by the Somogyi-Nelson method. As control, an enzyme liquid which has been inactivated by heating at 100.degree. C. for 10 minutes is treated similarly as above. One unit of the enzyme is defined as the amount of the enzyme that increases reducing power by one micromole for 1 minute in terms of the amount of glucose under the above

assay conditions.

DEPR:
To subject the solution of liquefied starch thus obtained either to non-reducing saccharide-forming enzyme and starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase or to non-reducing saccharide-forming enzyme, **trehalose**-releasing enzyme and starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase, pH and temperature are set to levels where these enzymes are active, in particular, pH4-10, desirably, pH5-8 and a temperature of about 10-80.degree. C., desirably, about 30-70.degree. C. There are however no limitations in the order of using the enzymes and they are successively or simultaneously used.

DEPR:
The amounts of enzymes to be used are arbitrarily chosen depending on reaction conditions including reaction time: Usually, against liquefied starch in solution, non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme are used in about 0.01-100 units/g solid; starch-debranching enzyme, about 1-10,000 units/g solid; and cyclomaltodextrin glucanotransferase, about 0.05-500 units/g solid. The less reducing saccharides thus obtained which contain non-reducing saccharides are characterized in that they contain large amounts of relatively small alpha-glycosyl trehaloses or alpha-glycosyl alpha-glycosides or **trehalose** because starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase acts on a solution of liquefied starch together with either non-reducing saccharide-forming enzyme or non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme. The wording "alpha-glycosyl alpha-glycoside" includes alpha-D-oligoglucosyl alpha-D-oligoglucosides which are disclosed in Japanese Patent Application No. 54,377/94 by the present applicant.

DEPR:
If necessary, one can degrade non-reducing saccharides bearing **trehalose** structures within their molecules or less reducing saccharides containing the same with alphaglucosidases or amylases, for example, alpha-amylase, betaamylase and glucoamylase so as to control their sweetening and reducing powers and/or to decrease their viscosities and, alternatively, hydrogenate the remaining reducing saccharides into saccharide alcohols so as to eliminate their reducing powers.

DEPR:
Especially, **trehalose** can be easily produced by subjecting non-reducing saccharides bearing **trehalose** structures within their molecules or less reducing saccharides containing the same to glucoamylase or alpha-glucosidase. Non-reducing saccharide or less reducing saccharide is subjected to glucoamylase or alpha-glucosidase into a solution of a mixture of **trehalose** and glucose which is then subjected to the above mentioned purification methods, for example, ion exchange column chromatography so as to remove glucose and also to recover **trehalose**-rich fractions. The fractions can be purified and concentrated into a syrup product which may be further concentrated to a supersaturated state and crystallized into crystalline **trehalose** hydrate or anhydrous crystalline **trehalose**.

DEPR:
To produce crystalline **trehalose** hydrate, for example, a high-**trehalose** content liquid, purity of about 60% or higher, concentration of about 65-90%, is placed in a crystallizer and gradually cooled at 95.degree. C. or lower, desirably, at 10-90.degree. C., if necessary, in the presence of 0.1-20% seed crystals to obtain a massecuite which contains crystalline **trehalose** hydrate. In this case, one can favorably employ a continuous crystallization method where **trehalose** is crystallized while concentrating under reduced pressure. Examples of methods which yield crystalline **trehalose** hydrate or saccharide mixture solid containing the same from such a massecuite include conventional crystal separation method, block pulverization method, fluidized-bed granulation method and spray drying method.

DEPR:
The crystal separation method is suitable to produce crystalline **trehalose** hydrate with an elevated purity, where massecuites are usually fed to a

basket-type centrifuge where they are separated into crystalline **trehalose** hydrate and mother liquor, after which the former crystals are sprayed with a minimum amount of chilled water for washing. In the spray drying method, massecuites, concentration of 70-85%, crystallizing ratio up to 20-60%, are usually sprayed through a nozzle combined with a high pressure pump, dried within a stream of hot air at a temperature where crystalline powder does not melt, for example, 60-100.degree. C. and aged in a stream of hot air, temperature of 30-60.degree. C., for about 1-20 hours, thus easily obtaining non- or less-hygroscopic crystalline mixture solids. In the block pulverization method, massecuites with moisture contents of 10-20%, crystallizing ratio up to 10-60%, are usually crystallized by allowing to stand for about 0.1-3 days into solids in block form which are then pulverized and dried by cutting or scraping, thus obtaining non- or less-hygroscopic crystalline mixture solids.

DEPR:
While to produce anhydrous crystalline **trehalose,** crystalline **trehalose** hydrate is converted by drying and, alternatively, a concentrated high**-trehalose** content liquid, moisture content less than 10%, is usually placed in a crystallizer and stirred at 50-160.degree. C., desirably, 80-140.degree. C., in the presence of seed crystals to obtain a massecuite which is then crystallized and pulverized, for example, by block pulverization method, fluidized-bed granulation method and spray drying method under relatively hot and dried conditions.

DEPR:
Further the saccharides are digested, absorbed and utilized as sources of calories when orally ingested because they are degraded by amylases, in particular, pancreas alpha-amylase, into small non-reducing oligosaccharides and small maltooligosaccharides which are readily degraded by alpha-glucosidase and small intestine enzymes to form glucose together with **trehalose** which is then degraded into glucose by trehalase. Still further the saccharides are feasible as sweetners less likely to cause caries because they are hardly fermented by dental caries-causative micro-organisms. Still further the saccharides bear other desirable properties such as osmosis controlling ability, shape imparting ability, gloss imparting ability, moisture retaining ability, viscosity, ability of preventing crystallization of other saccharides, decreased fermentability and ability of preventing retrogradation of gelatinized starch.

DEPR:
The **trehalose** according to the present invention can be favorably used for energy supplementation to living bodies because it is readily metabolized and utilized with no fears of toxicity or side effect when parenterally used in intubation feeding or infusion form. Crystalline high**-trehalose** content products can be favorably used as coating agents for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinyl pyrrolidone because **trehalose** acts as a stable sweetener.

DEPR:
Further the **trehalose** of the present invention can be used as a moisture-retaining agent, filler and viscosity-imparting agent in cosmetic cream, hair rinse, milky lotion and face lotion: In this case, when **trehalose** is used along with other moisture retaining agents, for example, propylene glycol, 1,3-butylene glycol, glycerin, sorbitol and polyoxyethylene oleil alcohol and vitamins such as alpha-glucosyl-L-ascorbic acid and enzyme-treated rutin, one can favorably obtain cosmetics with superior moisture retainabilities which include the ability to prevent spots and freckles due to ultraviolet radiation and also to whiten the skin.

DEPR:
Anhydrous crystalline **trehalose** can be favorably used as a desiccant for hydrous substances such as food products, cosmetics, medicines and materials and intermediates thereof to facilitate the production of stable and high-quality solid products including powders, granules and tablets.

DEPR:
Fifty milligrams of the non-reducing saccharide preparation PI, PII, PIII, PIV

or PV prepared in Experiment 4 was dissolved in 1 ml of 50 mM acetate buffer (pH4.5), added with one unit of the glucoamylase commercialized by Seikagaku Corp., Tokyo, Japan, incubated at 40.degree. C. for 6 hours for enzymatic hydrolysis and analyzed for degradation products on high-performance liquid chromatography, thus detecting glucose and **trehalose** as sole products. The noted glucose contents, **trehalose** contents and their molar ratios were as shown in in Table 5.

DEPR:
As evident from the results in Table 5, non-reducing saccharide PI was degraded by glucoamylase into one glucose molecule and one **trehalose** molecule; non-reducing saccharide PII, two glucose molecules and one **trehalose** molecule; non-reducing saccharide PIII, three glucose molecules and one **trehalose** molecule; non-reducing saccharide PIV, four glucose molecules and one **trehalose** molecule; and non-reducing saccharide PV, five glucose molecules and one **trehalose** molecule.

DEPR:
Considering the reaction characteristics of glucoamylase, these saccharides very likely bear structures where glucose molecule(s) is bound to **trehalose** molecule via alpha1,4 or alpha-1,6 linkage: Saccharide PI is a non-reducing saccharide with a glucose polymerization degree of 3 where one glucose molecule is bound to one **trehalose** molecule; saccharide PII, another non-reducing saccharide with a glucose polymerization degree of 4 where two glucose molecules are bound to one **trehalose** molecule; saccharide PIII, still another non-reducing saccharide with a glucose polymerization degree of 5 where three glucose molecules are bound to one **trehalose** molecule; saccharide PIV, still another non-reducing saccharide with a glucose polymerization degree of 6 where four glucose molecules are bound to one **trehalose** molecule; and saccharide PV, still another non-reducing saccharide with a polymerization degree of 7 where five glucose molecules are bound to one **trehalose** molecule. After subjecting to beta-amylase similarly as above, non-reducing saccharides PI and PII were not degraded; non-reducing saccharides PIII was degraded into one maltose molecule and one saccharide PI molecule; non-reducing saccharide PIV, into one maltose molecules and one saccharide PII molecule; and non-reducing saccharide PV, into two maltose molecules and one saccharide PI molecule.

DEPR:
The above evidence suggests that the reaction by the non-reducing saccharide-forming enzyme according to the present invention would be a type of intramolecular conversion reaction which accompanies neither degradation nor polymerization of substrates, in other words, accompanies no changes in glucose polymerization degrees, as well as suggesting that the non-reducing saccharides PI, PII, PIII, PIV and PV which are formed by the non-reducing saccharide-forming enzyme would be alpha-glucosyl **trehalose,** alpha-maltosyl **trehalose,** alpha-maltotriosyl **trehalose,** alpha-maltotetraosyl **trehalose** and alpha-maltopentaosyl **trehalose** respectively which can be commonly represented by the general formula "alpha-glycosyl **trehalose** (Gn-T where G, n and T mean glucose residue, an integer of 1 or more and alpha-**trehalose** respectively)".

DEPR:
While as evident from the results in Tables 7 and 8, saccharide preparations PI, PII, PIII, PIV and PV were found to be degradable by alpha-glucosidase and acetone-pulverized rat small intestine enzyme into glucose and **trehalose** as in the case of glucoamylase in Experiment 6.

DEPR:
The reaction products by alpha-glucosidase and acetone-pulverized rat small intestine enzyme were further subjected to the pig kidney trehalase commercialized by Sigma Chemical Co., St. Louis, Mo., USA, at pH5.7 and 37.degree. C. for 18 hours and then analyzed for saccharide composition on high-performance liquid chromatography, revealing that in saccharide preparations PI, PII, PIII, PIV and PV, the **trehalose** which had been formed by alpha-glucosidase or acetone-pulverized rat small intestine enzyme was degradable into glucose by the trehalase.

DEPR:
These results suggest that the non-reducing saccharide-forming enzyme according

to the present invention provides an entirely novel action mechanism where the reducing ends in reducing partial starch hydrolysates are intramolecularly converted into non-reducing **trehalose** structures.

DEPR:
The following will explain first **trehalose**-releasing enzymes from novel micro-organisms Rhizobium species M-11and Arthrobacter species Q36, then those from conventional micro-organisms.

DEPR:
The activity of non-reducing saccharide-forming enzyme in the culture was about 1.5 units/ml, while that of **trehalose**-releasing enzyme, about 2 units/ml. A portion of the culture was sampled and centrifugally separated into cells and supernatant and the cells were suspended in 50 mM phosphate buffer (pH7.0) to give the same volume as that of the sampled culture, followed by assaying enzymatic activities in the cell suspension and supernatant, revealing that there were found in the cell suspension about 0.6 units/ml non-reducing saccharide-forming enzyme and about 0.8 units/ml **trehalose**-releasing enzyme, while in the supernatant was found about 0.9 units/ml non-reducing saccharide-forming enzyme and about 1.2 units/ml **trehalose**-releasing enzyme.

DEPR:
Both non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme according to the present invention, which had been adsorbed on "DEAE TOYOPEARL", were eluted therefrom at different sodium chloride concentrations when a fresh preparation of the same buffer but additionally containing sodium chloride was passed through the column. The elution pattern from "DEAE TOYOPEARL" was as shown in FIG. 9. The non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme were eluted at sodium chloride concentrations of about 0.2M and 0.3M respectively and enzymatically active fractions for respective enzymes were separately recovered and purified.

DEPR:
Purification of the **trehalose**-releasing enzyme was carried out as follows: Fractions with **trehalose**-releasing emzyme activity eluted from "DEAE TOYOPEARL" were dialyzed against a fresh preparation of the same buffer but additionally containing 2M ammonium sulfate and applied to both hydrophobic and gel filtration column chromatography similarly as for the non-reducing saccharide-forming enzyme.

DEPR:
The enzymatic activities, specific activities and yields in respective purification stages were as shown in Table 11 for the non-reducing saccharide-forming enzyme and in Table 12 for the **trehalose**-releasing enzyme.

DEPR:
After testing both purified non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme for purity on electrophoresis using 7.5% polyacrylamide gel, they gave distinct single protein bands, suggesting that both enzyme preparations were electrophoretically homogenous and high in purity.

DEPR:
The purified **trehalose**-releasing enzyme obtained by the methods in Experiment 15 was electrophoresed on SDS-polyacrylamide gel, gel concentration of 10%, and then compared with the molecular weight markers commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan, which had been electrophoresed on the same gel, revealing that the molecular weight of the enzyme was about 58,000-68,000 daltons.

DEPR:
Alpha-glycosyl trehaloses as substrates were prepared in accordance with the method described in Japanese Patent Application No. 349,216/93. More particularly, 20% aqueous solutions of maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose as reducing partial starch hydrolysate were added with 2 units/g substrate solid of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 15, reacted at 40.degree. C. and pH7.0 for 48 hours, heated for inactivation,

filtered, deionized, concentrated in conventional manner and applied to ion exchange column chromatography on a strongly-acidic cation exchange "XT-1016" in sodium form. The ion exchange resin was packed in 3 jacketed-stainless steel columns, inner diameter of 2.0 cm, length of 1 m, and the columns were cascaded, loaded with 5 v/v % reaction saccharide liquid against the resin and injected with 55.degree. C. water at SV0.13 for fractionation while keeping the temperature inside the columns at 55.degree. C., thus obtaining high-purity preparations of non-reducing saccharides with glucose polymerization degrees of 3 or higher. Among these high-purity preparations, the glucosyl **trehalose** preparation had a purity of 97.6%; the maltosyl **trehalose** preparation, 98.6%, the maltotriosyl **trehalose** preparation, 99.6%; the maltotetraosyl **trehalose** preparation, 98.3%; and the maltopentaosyl **trehalose,** 98.1%.

DEPR:
20% Aqueous solutions of these five types of non-reducing saccharides or alpha-glycosyl trehaloses were prepared, added with 2 units/g substrate solid of the purified **trehalose**-releasing enzyme obtained in Experiment 15, reacted at 40.degree. C. and pH7.0 for 48 hours, deionized and subjected to high-performance liquid chromatography on "WAKO BEADS WB-T-330" for analysis of reaction products. As control, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were subjected to the purified **trehalose**-releasing enzyme similarly as above and then analyzed on high-performance liquid chromatography. The results were as shown in Table 13.

DEPR:
With these results, it would be concluded that the **trehalose**-releasing enzyme according to the present invention provides an entirely novel action mechanism where the linkages between the **trehalose** moieties and glycosyl moieties in alpha-glycosyl trehaloses are very specifically hydrolyzed to release **trehalose**.

DEPR:
In order to purify **trehalose** from respective reaction products, they were decolored, deionized, concentrated and subjected to column fractionation on a strongly-acidic cation exchange of sodium form "XT-1016", followed by recovering high-purity fractions with **trehalose** contents of 97% or higher. The fractions were then concentrated to about 65% and allowed to standing at 25.degree. C. for 2 days to crystallize out crystalline **trehalose** hydrate which was then separated and dried in vacuo to obtain a high-purity preparation with a **trehalose** content of 99% or higher. The yields against respective starting substrates on dry solid basis were as follows: from glycosyl **trehalose,** 9.5%; from maltosyl **trehalose,** 14.9%; from maltotriosyl **trehalose,** 16.0%; from maltotetraosyl **trehalose,** 18.5%; and from maltopentaosyl **trehalose,** 17.7%. The obtained **trehalose** was compared for melting point, melting heat, specific rotation, infrared absorption spectrum, powder x-ray diffraction pattern and degradation by the trehalase derived from the pig kidney commercialized by Sigma Chemical Co., St. Louis, Mo., USA, with the **trehalose** reagent as standard available from Wako Pure Chemical Industries, Ltd., Osaka, Japan, revealing that the high-purity **trehalose** preparations as tested exhibited a melting point of 97.0.+-.0.5.degree. C., a melting heat of 57.8.+-.1.2 KJ/mol and a specific rotation of +182.+-.1.degree. which were all in good agreement with those observed in the **trehalose** reagent, as well as that their infrared spectra and powder x-ray diffraction patterns were also in good agreement with those of the **trehalose** reagent. Further the high-purity **trehalose** preparations were degraded by the trehalase derived from pig kidney similarly as the **trehalose** reagent. As evident from the above results, the saccharides formed by subjecting alpha-glycosyl trehaloses to the **trehalose**-releasing enzyme were identified to be **trehalose**.

DEPR:
The reducing partial starch hydrolysates thus obtained and maltotriose as reducing partial starch hydrolysate with a glucose polymerization degree of 3 were diluted to 1% in 10 mM phosphate buffer (pH7.0), added with the purified non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme both prepared by the methods in Experiment 15 in respective amounts of 4 units/g substrate solid, reacted at 40.degree. C. for 24 hours, sampled in small portions, deionized and analyzed on high-performance liquid chromatography for

reaction products.

DEPR:
As shown in Table 14, the yields for **trehalose** from maltotriose by the non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme were low, i.e. 4.2%, while the reducing partial starch hydrolysates with glucose polymerization degrees of 10-34.1 marked high yields, i.e. 66.1-80.8%. Further it was revealed that higher the glucose polymerization degree of reducing partial starch hydrolysate as material, a higher **trehalose** purity was attainable. Also was revealed that the purity of **trehalose** was further increased by subjecting to glucoamylase the reaction mixture which has been exposed to both enzymes to degrade the residual alpha-glycosyl **trehalose** with glucose polymerization degrees of 3 or higher into **trehalose** and glucose.

DEPR:
10% High-purity **trehalose** preparation, purity of 99.5%, obtained by the method in Experiment 17 and 1% glycine in 50 mM phosphate buffer (pH7.0) was incubated at 100.degree. C. for 90 minutes, cooled and measured in 1cm cuvette for absorbance at 480 nm. As control, glucose and maltose were treated similarly as above and then measured for absorbance at 480 nm. The results were as shown in Table 15.

DEPR:
As evident from the results in Table 15, the **trehalose** preparation caused a trace coloration by Maillard reaction which was up to only 0.4-0.6% of those found in glucose and maltose, revealing that the **trehalose** preparation according to the present invention was a saccharide which would cause less Maillard reaction. Thus the saccharide less damage amino acids when mixed therewith.

DEPR:
In accordance with the method reported by Atsuji et al., Rinsho Eiyo (Clinical Nutrition), Vol.41, No. 2, pp.200-208 (1972), 30 g of a high-purity **trehalose** preparation, purity of 99.5%, obtained by the method in Experiment 17 was prepared into 20 w/v % aqueous solution and orally administered to healthy men, age of 26, 27, 28, 29, 30 or 31 years, after which their bloods were periodically sampled and measured for blood sugar and insulin levels. Glucose was used as control. As the result, **trehalose** behaved similarly as glucose did, and the blood sugar and insulin levels reached maxima about 0.5-1 hour after administration. Thus it was confirmed that **trehalose** was readily digested and absorbed and then metabolized and utilized as energy source.

DEPR:
An acute toxicity test was conducted on a high-purity **trehalose** preparation obtained by the method in Experiment 17, purity of 99.5%, where it was orally administered in mice. As the result, **trehalose** caused no death even in the possible highest dose. Thus its LD50 would be briefly 50 g/kg or higher.

DEPR:
Arthrobacter species Q36 (FERM BP-4316) in place of Rhizobium species M-11 (FERM BP-4130) was cultivated in fermenter for about 72 hours similarly as in Experiment 14. The activity of non-reducing saccharide-forming enzyme in the culture was about 1.3 units/ml, while that of the **trehalose**-releasing enzyme according to the present invention was about 1.8 units/ml. After assaying the enzymatic activities in the cell suspension and supernatant similarly as in Experiment 14, there were found in the cell suspension about 0.5 units/ml non-reducing saccharide-forming enzyme and about 0.5 units/ml **trehalose**-releasing enzyme, while in the supernatant were found about 0.8 units/ml non-reducing saccharide-forming enzyme and about 1.3 units/ml **trehalose**-releasing enzyme.

DEPR:
A culture, about 18 liters, obtained by the method in Experiment 22 was purified similarly as in Experiment 15. The results in respective purification stages were as shown in Table 16 for the non-reducing saccharide-forming enzyme and in Table 17 for the **trehalose**-releasing enzyme.

DEPR:

The purified non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme obtained as eluates of gel filtration in the stages in Tables 16 and 17 were electrophoresed similarly as in Experiment 15 for determination of purity, revealing that the protein bands were single and both purified enzymes were electrophoretically homogenous and high in purity.

DEPR:
After measuring on SDS-polyacrylamide gel electrophoresis similarly as in Experiment 16, the molecular weight of a purified **trehalose**-releasing enzyme obtained by the method in Experiment 23 was about 57,000-67,000 daltons. After testing the enzyme similarly as in Experiment 3, its isoelectric point was about 3.6-4.6. Effects of temperature and pH and thermal and pH stabilities of the enzyme were determined similarly as in Experiment 16. The results were as shown in FIG. 14 for the effect of temperature, in FIG. 15 for the effect of pH, in FIG. 16 for the thermal stability and in FIG. 17 for the pH stability.

DEPR:
A purified enzyme obtained by the method in Experiment 23 was tested for formation of **trehalose** from alpha-glycosyl trehaloses with glucose polymerization degrees of 3 or higher in accordance with the method in Experiment 17, revealing that the enzyme released **trehalose** from alpha-glycosyl trehaloses similarly as the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DEPR:
Among conventional micro-organisms, Brevibacterium helobolum (ATCC11822) and Micrococcus roseus (ATCC186) which had been confirmed for production of the **trehalose**-releasing enzyme according to the present invention were cultivated at 27.degree. C. in fermenter for 72 hours similarly as in Experiment 14. Respective cultures, about 18 liters each, were treated in cell disrupter and the supernatant was centrifugally recovered and subjected to salting out by ammonium sulfate, dialysis and ion exchange column chromatography in the given order, followed by characterizing the obtained partially purified enzyme preparations. The results are given in Table 18 along with those for Rhizobium species M-11 and Arthrobacter species Q36.

DEPR:
These partially purified enzymes derived from conventional micro-organisms were further tested in accordance with the method in Experiment 25 for formation of **trehalose** from alpha-glycosyl trehaloses with glucose polymerization degrees of 3 or higher, revealing that they released **trehalose** from alpha-glycosyl trehaloses similarly as the **trehalose**-releasing enzyme derived from Rhizobium species M-11.

DEPR:
To produce high-**trehalose** content saccharides from starch, effects of starch liquefaction degree and combinations of enzymes were studied. Cornstarch in 20% suspension was adjusted to pH6.5 by the addition of 0.1% calcium carbonate, added with 0.1-2.0% against starch solid of "TERMAMYL", an alpha-amylase commercialized by Novo Industri AS, Copenhagen, Denmark, reacted at 95.degree. C. for 15 minutes and kept at 120.degree. C. for 10 minutes in autoclave to obtain liquefied starch solutions with DE2.5-20.5 which were then immediately cooled, added with 5 units/g starch solid of a purified non-reducing saccharide-forming enzyme prepared by the method in Experiment 2, 10 units/g starch solid of a purified **trehalose**-releasing enzyme prepared by the method in Experiment 15 along with 500 units/g starch solid of isoamylase, a type of starch-debranching enzyme, and 5 units/g starch solid of cyclomaltodextrin glucanotransferase, both products of Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and reacted at pH6.0 and 45.degree. C. for 24 hours. The reaction mixtures were heated at 95.degree. C. for 10 minutes, cooled, added with 10 units/g starch solid of glucoamylase and reacted at pH5.0 for 10 hours. The reaction mixtures were analyzed on high-performance liquid chromatography to determine the **trehalose** contents (%) in the resultant saccharides. As control, fresh preparations of the same starch liquefied solutions were subjected only to the non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme similarly as above and then analyzed on high-performance liquid chromatography. The results were as shown in Table 19.

DEPR:
As evident from the results in Table 19, it was found that to produce high-**trehalose** content saccharides from starches, those with relatively low liquefaction degrees are preferred; desirably, those with DE lower than 15, more desirably, with DE lower than 10. As to the enzymes therefor, it was found that combination of non-reducing saccharide-forming enzyme, **trehalose**-releasing enzyme and starch debranching enzyme and/or cyclomaltodextrin glucanotransferase was very favorable in industrial-scale production of **trehalose** from starch rather than that of non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme because the former combination increased yields for **trehalose** from starch by about 2-4 folds.

DEPR:
The following Examples A are to illustrate the processes to produce non-reducing saccharide, less reducing saccharide containing the same and **trehalose** which are all according to the present invention, while Examples B are to illustrate compositions which contain the non-reducing saccharide, less reducing saccharides and/or **trehalose**.

DEPR:
Cornstarch in about 30% suspension was subjected to alpha-amylase in accordance with the method in Example A-3 into a liquefied starch solution with DE4 which was then added with 5 units/g starch solid of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 15 and 500 units/g starch solid of isoamylase and reacted at pH6.0 and 40.degree. C. for 48 hours. The reaction mixture contained 76.3% **trehalose** with respect to saccharide composition. The reaction mixture was then heated to inactivate the enzymes, decolored and deionized in conventional manner for purification, concentrated to about 85%, placed in a crystallizer, crystallized under stirring and gradually cooling conditions, distributed in plastic baths, allowed to standing at ambient temperature for 2 days and aged to complete crystallization to obtain solid products in block form. The products were then subjected to cutting machine to obtain a powder product of crystalline **trehalose** hydrate at the yield of 92% on dry solid basis. The product can be favorably used as sweetener, taste improving agent, quality improving agent, stabilizer and shape imparting agent in a variety of compositions including food products, cosmetics and medicines because it is substantially free of hygroscopicity and easily handleable.

DEPR:
Tapioca starch in about 30% suspension was subjected to alpha-amylase in accordance with the method in Example A-2 to obtain a liquefied starch solution with DE5 which was then added with 3 units/g starch solid of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 10 and 5 units/g starch solid of a purified **trehalose**-releasing enzyme obtained by the method in Experiment 23 along with 200 units/g starch solid of pullulanase and 3 units/g starch solid of cyclomaltodextrin glucanotransferase and reacted at pH6.0 and 45.degree. C. for 48 hours. The reaction mixture contained 84.7% **trehalose** on dry solid basis. The reaction mixture was then heated to inactivate the enzymes, decolored and deionized for purification in conventional manner and crystallized in continuous manner while concentrating, and the crystals in the resultant massecuite were separated by basket-type centrifuge and sprayed with a minimum amount of water for washing, thus obtaining a high-purity crystalline **trehalose** hydrate at the yield of about 55% on dry solid basis. The product, a crystalline **trehalose** hydrate with an extremely high purity, can be favorably used in a variety of compositions including food products, cosmetics and medicines, as well as reagent and material for industrial and chemical uses.

DEPR:
A heat-inactivated reaction mixture obtained by the method in Example A-6 was added with 10 units/g substrate solid of glucoamylase and reacted at pH5.0 and 50.degree. C. for 10 hours. The reaction mixture was heated to inactivate the enzyme, decolored and deionized for purification in conventional manner, concentrated to about 70%, placed in crystallizer and crystallized under stirring and gradually cooling conditions to obtain a massecuite with a crystallization degree of about 40%. The massecuite was then sprayed at 150 kg/cm.sup.2 through a high-pressure nozzle provided at the top of a drying

tower, while supplying downwards 85.degree. C. air from the top of the drying tower and collecting the resultant crystalline powder on a conveyer of metal net provided at the bottom of the drying tower. The crystalline powder was gradually moved and transferred outside the drying tower while supplying 45.degree. C. air upwardly through the conveyer. The crystalline powder was placed in ageing tower where the powder was aged for 10 hours in a stream of warmed air to complete crystallization and drying, thus obtaining a crystalline **trehalose** hydrate product at the yield of about 87% against material starch solid. The product can be favorably used as sweetener, taste improving agent, quality improving agent, stabilizer and shape imparting agent in a variety of compositions including food products, cosmetics and medicines because it is substantially free of hygroscopicity and easily handleable.

DEPR:
A mutant of Rhizobium species M-11 (FERM BP-4130) was cultivated for about 70 hours in accordance with the method in Experiment 1. The culture was passed through SF membrane to remove the cells and the filtrate, about 100 liters, was then passed through UF membrane to obtain 5 liters of a concentrate which contained about 410 units/ml non-reducing saccharide-forming enzyme and about 490 units/ml **trehalose**-releasing enzyme. Cornstarch in about 33% suspension was subjected to alpha-amylase in accordance with the method in Example A-3 to obtain a liquefied starch solution with about DE4 which was then added with 0.02 ml/g starch solid of the above concentrate, 500 units/g starch solid of isoamylase and 5 units/g starch solid of cyclomaltodextrin glucanotransferase and reacted at pH6.2 and 40.degree. C. for 48 hours. The reaction mixture was heated to inactivate the enzymes, added with 10 units/g substrate solid of glucoamylase and further reacted at pH5.0 and 50.degree. C. for 10 hours. The reaction mixture contained 85.6% **trehalose** on dry solid basis. The reaction mixture was then heated to inactivate the enzyme, decolored and deionized for purification in conventional manner and crystallized in continuous manner while concentrating, and the crystals in the resultant massecuite were separated with basket-type centrifuge and sprayed with a minimum amount of water for washing, thus obtaining a high-purity crystalline **trehalose** hydrate at the yield of 64% on dry solid basis. The product, a crystalline **trehalose** hydrate with an extremely high purity, can be favorably used in a variety of compositions including food products, cosmetics and medicines, as well as reagent and material for industrial and chemical uses.

DEPR:
A reaction mixture obtained by the method in Example A-8 was heated to inactive enzymes, decolored and deionized for purification in conventional manner and concentrated to obtain a 55% syrup product. The product was applied as starting saccharide liquid to column chromatography on "DOWEX 99", a strongly-acidic cation exchange of calcium form, crosslinkage degree of 6%, commercialized by The Dow Chemical Co., Midland, Mich., USA, to elevate the content for **trehalose,** followed by recovering **trehalose**-rich fractions. The fractions were then decolored and deionized in conventional manner, placed in evaporator and boiled in vacuo into a syrup with a moisture content of about 3.0%. The syrup was placed in crystallizer, added with 1% anhydrous crystalline **trehalose** as seed crystal against syrup solid, crystallized at 120.degree. C. while stirring, distributed in aluminum baths and aged at 100.degree. C. for 6 hours to obtain solid products in block form. The solid products were then subjected to cutting machine and dried while fluidizing to obtain a powder product of anhydrous crystalline **trehalose** with a moisture content of about 0.3% at the yield of about 75% against the solid in the **trehalose**-rich fractions. The product can be favorably used as desiccant for hydrous substances such as food products, cosmetics, medicines and their materials and intermediates, as well as sweetener with an gentle sweetness in a variety of compositions including food products, cosmetics and medicines.

DEPR:
One part by weight of a crystalline **trehalose** hydrate powder obtained by the method in Example A-7 was mixed to homogeneity with 0.01 part by weight of "ALPHA G SWEET", an alpha-glycosyl stevioside commercialized by Toyo Sugar Refining Co., Ltd., Tokyo, Japan, and 0.01 part by weight of "ASPARTAME", an L-aspartyl-L-phenylalanine methyl ester commercialized by Ajinomoto Inc., Tokyo, Japan, and the mixture was fed to granulater to obtain a granular product of sweetener. The product has a superior quality for sweetness and a

sweetening power about 2-fold stronger than that of sucrose and its calorie is about one half of that of sucrose per sweetening power. Since the product is superior in stability and free of decomposition of the ingredients which exhibit high sweetening powers, it is suitable as low-calorie sweetener to sweeten low-calorie food products for persons with diabetes or obesity whose calorie intakes are restricted. Further the product is suitable to sweeten food products which are suppressive on dental caries because it induces less formation of acids and insoluble glucans by dental caries-causative micro-organisms.

DEPR:
Forty parts by weight of cacao paste, 10 parts by weight of cacao butter, 30 parts by weight of sucrose, 20 parts by weight of a high-purity crystalline trehalose hydrate obtained by the method in Example A-8 were mixed, fed to refiner to reduce particle sizes and kneaded in conche at 50.degree. C. for 2 days. During the kneading, 0.5 parts by weight of lecithin was added and sufficiently dispersed to homogeneity. The resultant was adjusted to 31.degree. C. with thermocontroller, distributed in molds immediately before solidification of the butter, deaerated with vibrator and passed through 10.degree. C. cooling tunnel over 20 minutes for solidification. The contents were taken out from the molds and packaged to obtain products. The products are free of hygroscopicity but have a superior color, gloss and texture and smoothly dissolves in the mouth to give a gentle sweetness and a mild flavor and taste.

DEPR:
Three parts by weight of gum base was softened by melting while heating, added with 4 parts by weight of sucrose and 3 parts by weight of a crystalline trehalose hydrate powder obtained by the method in Example A-5, mixed with appropriate amounts of flavoring and coloring agents, kneaded with roller in conventional manner, shaped and packaged to obtain a product. The product is a chewing gum with a superior texture, flavor and taste.

DEPR:
Thirty three parts by weight of orange juice powder prepared by spray drying was mixed by stirring to homogeneity with 50 parts by weight of a high-purity crystalline trehalose hydrate obtained by the method in Example A-6, 10 parts by weight of sucrose, 0.65 parts by weight of anhydrous citric acid, 0.1 part by weight of malic acid, 0.1 part by weight of L-ascorbic acid, 0.1 part by weight of sodium citrate, 0.5 parts by weight of pullulan and an appropriate amount of powdered flavoring agent, cut into fine powder, fed to fluidized-bed granulator, sprayed at an exhausting temperature of 40.degree. C. with a syrup as binder which had been obtained by purifying and concentrating a high-trehalose content reaction mixture obtained by the method in Example A-6, granulated for 30 minutes, divided into prescribed amounts and packaged to obtain a product. The product is a powdered juice with a fruit juice content of about 30%. The product was free of unpleasant taste and odor and stable over an extended time period.

DEPR:
Ninety parts by weight of rice powder was mixed to homogeneity with 20 parts by weight of cornstarch, 40 parts by weight of sucrose, 80 parts by weight of a crystalline trehalose hydrate powder obtained by the method in Example A-5 and 4 parts by weight of pullulan to obtain "uiro-no-moto". The uiro-no-moto was then kneaded to homogeneity with appropriate amounts of "matcha" or powdered green tea in water, placed in vessels and steamed for 60 minutes to obtain "matcha-uiro". The product is superior in gloss, texture, taste and flavor. The product has an extended shelf life because retrogradation of starch is effectively suppressed.

DEPR:
One part by weight of "HIMUTE S", a soybean peptide in 40% solution directed to use in food products commercialized by Fuji Oil Co., Ltd., Osaka, Japan, was mixed with 2 parts by weight of a high-purity crystalline trehalose hydrate obtained by the method in Example A-6, placed in plastic baths, dried at 50.degree. C. in vacuo and cut to obtain a powder product of peptide. The product, which is superior in taste and flavor, can be favorably used as material in confectioneries such as mixes and ice desserts, as well as babies'

foods and nutriment for therapeutic uses including oral and parenteral liquid foods.

DEPR:
One part by weight of red miso was mixed with 3 parts by weight of an anhydrous crystalline **trehalose** powder obtained by the method in Example A-9, poured in a plurality of concaves on metal plate, allowed to stand at ambient temperature overnight for solidification and put off from the plate to obtain miso solids, about 4 g each, which were then fed to cutting machine into powder. The product can be favorably used as seasoning in convenient Chinese-style noodles and "suimono", a type of clear soup. While the miso solids can be used intact as confectionery, as well as solid seasoning.

DEPR:
Raw egg york was pasteurized at 60-64.degree. C. on plate heater and the obtained egg york liquid was mixed with 4 parts by weight of an anhydrous crystalline **trehalose** powder obtained by the method in example A-9 against one part by weight of the egg york liquid, distributed in baths and allowed to stand overnight to convert the **trehalose** into crystalline hydrate form, thus obtaining solid products of block form. The solid products were then fed to cutting machine to obtain a powdered egg york. The product can be favorably used as material for confectioneries such as mixes, ice desserts and emulsifier, as well as babies' food and nutriment for therapeutic uses including oral and parenteral liquid foods.

DEPR:
One part by weight of **trehalose** obtained by the method in Example A-6, 2 parts by weight of alpha-glucosyl-L-ascorbic acid commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 2 parts by "ALPHA G RUTIN", an enzyme-treated rutin commercialized by Toyo Sugar Refining Co., Ltd., Tokyo, Japan, 2 parts by weight of distearic methyl ammonium chloride, 2 parts by cetanol, 2 parts by weight of silicone oil, 1 part by weight of polyoxyethylene oleil alcohol ether and an appropriate amount of flavoring agent were dissolved while heating, added with a mixture of 3 parts by weight of 1,3-butylene glycol, 89 parts by weight of refined water and an appropriate amount of antiseptic while stirring, cooled and allowed to standing to obtain a hair rinse. The product with alpha-glucosyl-L-ascorbic acid and enzyme-treated rutin can be favorably used to stimulate the generation and growth of hair in humans and animals, as well as to treat and prevent scurf, itching and fallen hair.

DEPR:
One half part by weight of ginseng extract was kneaded together with 1.5 parts by weight of an anhydrous crystalline **trehalose** powder obtained by the method in Example A-9, placed in baths and allowed to stand for 2 days to convert the **trehalose** into crystalline hydrate form, thus obtaining solid products in block form. The solid products were then fed to cutting machine for pulverization and sieved to obtain a powdered ginseng extract. The powder was fed to granulator together with appropriate amounts of vitamin B1 and vitamin B2, both in powder, into a granular ginseng extract containing vitamins. The product can be favorably used as tonic. Further the product can be also used as hair restorer.

DEPR:
A natural human interferon-alpha preparation commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was applied to an immobilized anti-human interferon-alpha antibody column in conventional manner so as to adsorb the human interferon-alpha and also to pass through the bovine serum albumin as stabilizer, and the adsorbed natural human interferon-alpha was eluted with a physiological saline containing 5% high-purity crystalline **trehalose** hydrate obtained by the method in Example A-6 while changing the pH level in the saline. The obtained liquid was filtered through membrane, added with about 20-fold amount of "FINETOSE", an anhydrous crystalline maltose commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, for desiccation, pulverized and fed to tabletting machine to obtain tablets, about 200 mg each, which contained about 150 units of natural human interferon-alpha per tablet. The product can be favorably used as sublingual tablet in the treatment of viral diseases, allergic diseases, rheumatism,

diabetes and malignant tumors where the product is orally administered in a dose of 1-10 tablets/day/adult. Especially the product can be favorably used in the treatment of AIDS and hepatitis whose incidences have been rapidly increasing in these years. The product retains its activities over an extended time period even when allowed to standing at ambient temperature because both non-reducing saccharide according to the present invention and anhydrous crystalline maltose act as stabilizers.

DEPR:
Non-coated tablets as core material, 150 mg each, were coated with an undercoating liquid consisting of 40 parts by weight of a high-purity crystalline **trehalose** hydrate obtained by the method in Example A-8, 2 parts by weight of pullulan with an averaged molecular weight of 200,000 daltons, 30 parts by weight of water, 25 parts by weight of talc and 3 parts by weight of titanium oxide to give about 230 mg per tablet, further coated with a final coating liquid consisting of 65 parts by weight of the same crystalline **trehalose** hydrate, one part by weight of pullulan and 34 parts by weight of water and polished with liquid wax to obtain sugar-coated tablets with superior glossy appearance. The product has a superior shock resistance and retains a high-quality over an extended time period.

DEPR:
A composition consisting of 500 parts by weight of a crystalline **trehalose** hydrate powder prepared by the method in Example A-7, 270 parts by weight of powdered egg york, 4.4 parts by weight of sodium chloride, 1.8 parts by weight of potassium chloride, 4 parts by weight of magnesium sulfate, 0.01 part by weight of thiamin, 0.1 part by weight of sodium ascorbate, 0.6 parts by weight of vitamin E acetate and 0.04 parts by weight of nicotinamide was divided into 25 g aliquots which were then packed in moisture-proof laminated bags and heat-sealed to obtain a product. One bag of the product is dissolved in about 150-300 ml water into a liquid food which is then administered in the oral or nasal cavity, stomach or intestine for energy supplementation to living bodies.

DEPR:
A high-purity crystalline **trehalose** hydrate produced by the method in Example A-8 was dissolved in water to about 10 w/v %, passed through membrane to remove pyrogens, sterilely bottled in plastic bottles and sealed in conventional manner. The product is a stable infusion agent which is free of alteration in time course and suitable for intravenous and intraperitoneal administration. The product is isotonic at 10 w/v % to blood and therefore capable of supplementing at the concentration 2-fold more energy than in case of using glucose.

DEPR:
A high-purity crystalline **trehalose** hydrate obtained by the method in Example A-8 and an amino acid mixture with the below described formulation were mixed and dissolved in water to 5 w/v % and 30 w/v % respectively, purified similarly as in EXAMPLE B-25 to remove pyrogens, distributed in plastic bags and sealed.

DEPR:
The product is a stable infusion agent which is free of alteration in time course and favorably administrable through intravenous and intraperitoneal routes because **trehalose** exhibits no reducing power even in this type of composition of saccharide and amino acid. The procut can be favorably used to supplement both energy and amino acids to living bodies.

DEPR:
Two hundred parts by weight of a crystalline **trehalose** hydrate powder prepared by the method in Example A-5 and 300 parts by weight of maltose were first admixed with 3 parts by weight of iodine in 50 parts by weight of methanol, then with 200 parts by weight of 10 w/v % aqueous pullulan solution, thus obtaining an ointment with an appropriate extensibility and adhesiveness. The use of the product superiorly heals external injuries in a shortened treatment period because the iodine and **trehalose** in the product act as disinfectant and energy supplementing agent to viable cells respectively.

DEPR:
As obvious from the above explanation, in the production of non-reducing

saccharides with **trehalose** structures such as alpha-glycosyl trehaloses and alpha-glycosyl alpha-glycosides and less reducing saccharides containing the same from starch, combination of non-reducing saccharide-forming enzyme and starch debranching enzyme and/or cyclomaltodextrin glucanotransferase improves the yields for non-reducing saccharides from liquefied starches in solution, as well as facilitating the industrial-scale production of relatively small less reducing saccharides with decreased viscosity and superior handleability. Further in the production of **trehalose** from starch, combination of non-reducing saccharide-forming enzyme, **trehalose**-releasing enzyme and either of both of starch debranching enzyme and cyclomaltodextrin glucanotransferase greatly improves the yield for **trehalose** from starch and facilitates its industrial-scale production. The non-reducing saccharides including alpha-glycosyl **trehalose** and alpha-glycosyl alpha-glycoside and **trehalose** and less reducing saccharides containing the same bear a superior stability and a high-quality, mild sweetness. Still further they are digested and absorbed as calorie source when orally intaken. **Trehalose** would also find parenteral uses where it is readily metabolized and assimilated. Thus the non-reducing saccharides and less reducing saccharides containing the same both according to the present invention can be favorably used as sweeteners, taste improving agents, quality improving agents, stabilizers and shape imparting agents in a variety of compositions including food products, cosmetics and medicines.

DEPL:
Production of **trehalose**-releasing enzyme from Rhizobium species M-11

DEPL:
Properties of **trehalose**-releasing enzyme

DEPL:
Preparation of **trehalose** from alpha-glycosyl **trehalose**

DEPL:
Preparation of **trehalose** from reducing partial starch hydrolysates

DEPL:
Production of **trehalose**-releasing enzyme from Arthrobacter species Q36

DEPL:
Preparation of **trehalose** from alpha-glycosyl **trehalose**

DEPL:
Production and Properties of **trehalose**-releasing enzyme from conventional micro-organisms

DEPL:
Effects of starch liquefaction degree and types of enzymes on the production of high-**trehalose** content saccharides

DEPV:
(1) Action Capable of specifically hydrolyzing the linkages between the **trehalose** moieties and the other moieties in alpha-glycosyl trehaloses.

DEPV:
(2) Non-reducing saccharide PV yields non-reducing saccharide PII and maltotriose as predominant products when subjected to alpha-amylase, while non-reducing saccharide PII, one **trehalose** molecule and two glucose molecules when subjected to glucoamylase.

DEPV:
(1) The **trehalose**-releasing enzyme specifically hydrolyzes the linkages between **trehalose** moieties and glycosyl moieties in alpha-glycosyl trehaloses to form **trehalose** and reducing saccharides with glucose polymerization degrees of 1 or higher.

DEPV:
(2) Maltooligosaccharides are not susceptible at all to the **trehalose**-releasing enzyme.

DETL:
TABLE 5 Saccharide Glucose **Trehalose** Molar ratio Preparation (%) (%) (glucose/trehalose) PI 36.2 63.8 1.07 PII 52.0 48.0 2.06 PIII 61.4 38.6 3.02 PIV 68.3 31.7 4.09 PV 72.9 27.1 5.11

DETL:
TABLE 7 Composition in degradation product Saccharide by alpha-glucosidase (%) preparation Glucose **Trehalose** Others PI 36.5 63.0 0.5 PII 52.1 47.6 0.3 PIII 61.7 38.1 0.2 PIV 69.5 30.2 0.3 PV 71.4 28.3 0.3

DETL:
TABLE 8 Composition in degradation product by Saccharide acetone-powdered rat small intestine enzyme (%) preparation Glucose **Trehalose** Others PI 37.2 62.4 0.4 PII 52.5 47.1 0.4 PIII 62.0 37.6 0.4 PIV 68.8 30.8 0.4 PV 73.4 26.5 0.1

DETL:
TABLE 13 Elution time Composition Substrate Reaction product on HPLC (min) (%) Glucosyl **Trehalose** 27.4 17.5 **trehalose** Glucose 33.8 6.5 Glucosyl 23.3 76.0 **trehalose** Maltosyl **Trehalose** 27.4 44.3 **trehalose** Maltose 28.7 44.4 Maltosyl 21.6 11.3 **trehalose** Maltotriosyl **Trehalose** 27.4 39.5 **trehalose** Maltotriose 25.9 60.0 Maltotriosyl 19.7 0.5 **trehalose** Maltotetraosyl **Trehalose** 27.4 34.2 **trehalose** Maltotetraose 24.1 65.5 Maltotetraosyl 18.7 0.3 **trehalose** Maltopentaosyl **Trehalose** 27.4 29.1 **trehalose** Maltopentaose 22.6 70.6 Maltopentaosyl 17.8 0.3 **trehalose** Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

DETL:
TABLE 14

Glucose polymerization degree of reducing Composition (%) partial starch After reaction by After reaction hydrolysate Reaction product NRSF and TR enzymes by glucoamylase 34.1 **Trehalose** 80.8 83.5 Glucose 0.2 16.5 Reducing oligosaccharides 14.4 0.0 Glycosyl trehaloses 4.6 0.1 26.2 **Trehalose** 79.7 82.5 Glucose 0.2 17.5 Reducing oligosaccharides 15.3 0.0 Glycosyl trehaloses 4.8 0.0 18.1 **Trehalose** 77.7 80.7 Glucose 0.2 19.3 Reducing oligosaccharides 17.0 0.0 Glycosyl trehaloses 5.1 0.0 15.2 **Trehalose** 75.0 78.5 Glucose 0.3 21.5 Reducing oligosaccharides 18.6 0.0 Glycosyl trehaloses 6.1 0.0 10.0 **Trehalose** 66.1 70.1 Glucose 0.3 29.9 Reducing oligosaccharides 27.6 0.0 Glycosyl trehaloses 7.7 0.0 3 **Trehalose** 4.2 20.8 (Maltotriose) Glucose 2.1 79.2 Maltotriose 65.0 0.0 Glucosyl **trehalose** 28.7 0.0

Note: In the Table, NRSF and TR enzymes mean nonreducing saccharideformin and trehalosereleasing enzymes respectively. Glycosyl **trehalose** means nonreducing saccharides with glucose polymerization degrees of 3 or higher.

DETL:
TABLE 15 Saccharide Coloration degree preparation as tested at 480 nm Remarks **Trehalose** 0.006 Present invention Glucose 1.671 Control Maltose 0.926 Control

DETL:
POE (20) cetyl alcohol ether 1 "SILICONE KF96" commercialized by the Shin-etsu 2 Chemical Industry Co., Ltd., Tokyo, Japan Liquid paraffin 5 Propylene glycol 1 Glycerin 1 **Trehalose** obtained in Example A-7 1 Ethyl alcohol 15 Caboxyvinyl polymer 0.3

Hydroxypropyl cellulose 0.1 2-Aminomethyl propanol 0.1 POE in castor oil 0.1 Alpha-glucosyl-L-ascorbic acid 1 Red Pigment No. 106 0.0001 Antiseptic trace Flavoring agent 0.1 Distilled water 70

DETL:

Sorbitol 2 **trehalose** obtained in Example A-8 0.5 Placeta liquid 0.5 Alpha-glucosyl-L-ascorbic acid 0.5 Dimethyl stearylamine oxide 0.05 Sodium laurate 0.01 Ethyl alcohol 20 Antiseptic trace Flavoring agent trace Distilled water 75

DETL:

Calcium hydrogen phosphate 45.0 Pullulan 2.95 Sodium lauryl sulfate 1.5 Glycerin 20.0 Polyoxyethylene sorbitan laurate 0.5 Antiseptic 0.05 Crystalline **trehalose** hydrate powder 12.0 obtained by the method in Example A-5 Maltitol 5.0 Water 13.0

CLPR:
1. A non-reducing saccharide having a **trehalose** structure wherein at least one molecule of glucose is attached to each of the glucose molecules making up the **trehalose** structure which is obtainable by subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase, or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase; and collecting the produced non-reducing saccharide.

CLPR:
5. A process for producing a non-reducing saccharide having a **trehalose** structure or a saccharide composition containing the same, wherein said non-reducing saccharide has at least one molecule of glucose attached to each of the glucose molecules making up the **trehalose** structure, said process comprising:

CLPR:
7. A process for producing a non-reducing saccharide having a **trehalose** structure or a saccharide composition containing the same, said non-reducing saccharide having at least one molecule of glucose attached to each of the glucose molecules making up the **trehalose** structure, such process comprising:

CLPR:
9. In a method for producing a non-reducing saccharide having a **trehalose** structure wherein at least one molecule of glucose is attached to each of the glucose molecules making up the **trehalose** structure, wherein a solution of liquified starch is subjected either to a non-reducing saccharide-forming enzyme or to a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme, the improvement comprising elevating the yield of such non-reducing saccharide, comprising allowing a starch-debranching enzyme and cyclomaltodextrin glucanotransferase and either or both of the non-reducing saccharide enzyme and the **trehalose**-releasing enzyme to act on said solution.

CLPR:
16. The process according to claim 5 wherein said saccharide composition comprises said non-reducing saccharide and at least one member selected from the group consisting of non-reducing saccharides bearing at the end a **trehalose** structure, non-reducing saccharides bearing within the molecule a **trehalose** structure, and **trehalose**.

CLPR:
17. The process of claim 16 wherein said **trehalose** is crystalline **trehalose** hydrate or anhydrous crystalline **trehalose**.

CLPR:
18. The process of claim 7 wherein said saccharide composition comprises said non-reducing saccharide and at least one member selected from the group consisting of non-reducing saccharides bearing at the end a **trehalose** structure and **trehalose**.

CLPR:
19. The process of claim 18 wherein said **trehalose** is crystalline **trehalose** hydrate or anhydrous crystalline **trehalose**.

CLPR:
20. The method of claim 9 wherein said saccharide composition comprises said non-reducing saccharide and at least one saccharide selected from the group consisting of **trehalose** and non-reducing saccharides bearing at the end a **trehalose** structure.

CLPR:
21. The method of claim 20 wherein said **trehalose** is crystalline **trehalose** hydrate or anhydrous crystalline **trehalose**.

CLPR:
22. The saccharide composition of claim 4, wherein said other saccharides are selected from the group consisting of **trehalose** and non-reducing saccharides bearing at the end a **trehalose** structure.

CLPV:
subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme and a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase to form such non-reducing saccharide; and

CLPV:
subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme, and a starch-debranching enzyme and cyclomaltodextrin glucanotransferase;

CLPV:
subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase, or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme, a starch-debranching enzyme and cyclomaltodextrin glucanotransferase to form a non-reducing saccharide having a **trehalose** structure wherein at least one molecule of glucose is attached to each of the glucose molecules making up the **trehalose** structure;

CLPV:
subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase, or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme, a starch-debranching enzyme and cyclomaltodextrin glucanotransferase to form a non-reducing saccharide having a **trehalose** structure wherein at least one molecule of glucose is attached to each of the glucose molecules making up the **trehalose** structure;

CLPV:
subjecting a solution of liquified starch to a non-reducing saccharide-forming enzyme, a starch-debranching enzyme, and cyclomaltodextrin glucanotransferase, or to a non-reducing saccharide-forming enzyme, a **trehalose**-releasing enzyme, a starch-debranching enzyme and cyclomaltodextrin glucanotransferase to form a non-reducing saccharide having a **trehalose** structure wherein at least one molecule of glucose is attached to each of the glucose molecules making up the **trehalose** structure;

ORPL:
Hoelzle et al, Increased Accumulation of **Trehalose** in Rhizobia . . . vol. 56, No. 10, pp. 3213-3215, Oct. 1990.

USPT

US-CL-CURRENT: 426/658,435/100 ,435/72 ,435/99 ,514/54 ,514/61 ,514/777 ,536/1.11 ,536/123.1 ,536/124 ,536/4.1

US-PAT-NO: 5863771
DOCUMENT-IDENTIFIER: US 5863771 A
TITLE: Saccharide composition comprising maltooligosylturanose and maltooligosylpalatinose, its preparation and uses
DATE-ISSUED: January 26, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Aga; Hajime	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Fukuda; Shigeharu	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/101,426/658 ,435/100 ,435/72 ,435/99 ,514/54 ,514/61 ,514/777 ,536/1.11 ,536/123.1 ,536/124 ,536/4.1
ABSTRACT:
A saccharide composition comprising maltooligosyl derivatives of turanose and palatinose which can be readily produced, separated, and purified in a relatively-high yield from maltooligosylsucrose by allowing non-reducing saccharide-forming enzymes to act on aqueous solutions containing maltooligosylsucrose. These saccharides are reducing oligosaccharides with a mild and high-quality sweetness and can be used orally and parenterally, as well as being readily metabolized and used by living bodies.
17 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

DEPR:
As is disclosed in Japanese Patent Laid-Open No. 143,876/95, the non-reducing saccharide-forming enzymes usable in the present invention are produced from Rhizobium sp. M-11 (FERM BP-4130), Arthrobacter sp. Q36 (FERM BP-4316), Brevibacterium helvolum (ATCC 11822), Flavobacterium aquatile (IFO 3772), Micrococcus roseus (ATCC 186), Curtobacterium citreum (IFO 15231), Mycobacterium smegmatis (ATCC 19420), Terrabacter tumescens (IFO 12960), and other microorganisms of the genus **Sulfolobus** as disclosed in Japanese Patent Application No. 166,011/94. These enzymes are intramolecular saccharide-transferring enzymes which convert or rearrange maltooligosaccharides into maltooligosyltrehalose. The equilibrium point of these enzymes predominantly inclines to the side of forming maltooligosyltrehalose: For example, they produce maltotriosyltrehalose from maltopentaose as a substrate in a yield of at least about 90 w/w %, on a dry solid basis (the wording "w/w %, on a dry solid basis" as referred to in the present invention will be abbreviated as "%", unless specified otherwise).

DEPR:
In addition to the above enzymes, other enzymes usable in the present invention are those which are derivable from microorganisms of the genera Rhizobium, Arthrobacter, Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium, Mycobacterium, Terrabacter, and **Sulfolobus,** as well as their mutants capable of producing the desired enzymes and those obtained from transformed microorganisms into which genes coding for the enzymes are introduced, can be selectively used. For example, microorganisms of the genus **Sulfolobus** such as strains of **Sulfolobus acidocaldarius** (ATCC 33909 and 49426) and **Sulfolobus** solfataricus (ATCC 35091 and 35092) can be advantageously used.

DEPR:
Any synthetic and natural nutrient culture media can be used for culturing the above microorganisms as long as they grow therein and produce the desired enzymes. The carbon sources usable in the present invention include those which can be assimilated by the microorganisms: For example, saccharides such as glucose, fructose, molasses, **trehalose,** lactose, sucrose, mannitol, sorbitol, and partial starch hydrolysates, and inorganic acids such as citric acid and succinic acid, as well as their salts, can be used. The concentration

of these carbon sources is appropriately chosen depending on their types. For example, in the case of using glucose as a carbon source, a preferable concentration is 40 w/v % or lower, more particularly, a concentration of 10 w/v % or lower is more preferably used with respect to the microorganisms' growth and proliferation. The nitrogen sources usable in the present invention are, for example, inorganic nitrogen-containing compounds such as ammonium salts and nitrates, and organic nitrogen-containing compounds such as urea, corn steep liquor, casein, peptone, yeast extract, and meat extract. The inorganic ingredients usable in the present invention are, for example, salts of calcium, magnesium, potassium, sodium, phosphoric acid, manganese, zinc, iron, copper, molybdenum, and cobalt.

DEPR:
The method for assaying the activity of the non-reducing saccharide-forming enzymes used in the present invention is as follows: Add one ml of an enzyme solution to 4 ml of 1.25 w/v % maltopentaose as a substrate dissolved in 50 mM phosphate buffer (pH 7.0), react the mixture at 40.degree. C. for 60 min, heat the reaction mixture at 100.degree. C. for 10 min to suspend the enzymatic reaction, precisely dilute the mixture with deionized water by 10-fold, and assay the reducing power of the dilution by the Somogyi-Nelson's method. As a control, an enzyme solution which had been heated at 100.degree. C. for 10 min to inactivate the enzyme is assayed similarly as above. One unit activity of the enzyme is defined as the amount of enzyme which eliminates the reducing power of that of one micromole of maltopentaose per minute when assayed by the above method. In the case of non-reducing saccharide-forming enzymes from microorganisms of the genus **Sulfolobus,** the enzymes are reacted at 60.degree. C. and pH 5.5, then inactivated by heating at 100.degree. C. for 30 min.

DEPR:
In accordance with the methods in Experiments 2 and 3, purified non-reducing saccharide-forming enzymes from Arthrobacter sp. Q36 (FERM BP-4316) and Rhizobium sp. M-11 (FERM BP-4130) were prepared, and a partially purified non-reducing saccharide-forming enzyme from **Sulfolobus acidocaldarius** (ATCC 33909) was obtained by column chromatography using "DEAE-TOYOPEARL.RTM. 650". These enzymes were allowed to act on a 20% maltotetraosylsucrose solution for 96 hours under the conditions as shown in Table 2. The results were in Table 2.

DEPR:
One hundred ml aliquots of a liquid nutrient culture medium, consisting of 0.1 w/v % peptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, were distributed into 500-ml Erlenmeyer flasks, sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to pH 3.0 by the addition of sulfuric acid. The resulting culture medium was inoculated with a stock culture of **Sulfolobus acidocaldarius** (ATCC 33909) and cultured at 75.degree. C. for 24 hours under shaking conditions of 130 rpm. The culture thus obtained was used as a first seed culture.

DEPR:
Two and half parts by weight of sucrose and 2.5 parts by weight of maltopentaose commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, were dissolved by heating in 5 parts by weight of water, and the solution was adjusted to pH 6.5 and 45.degree. C., admixed with one unit/g sucrose of levansucrase from a strain of the genus Bacillus, enzymatically reacted for 20 hours, and heated to inactivate the remaining enzyme. The reaction mixture was in a conventional manner decolored with an activated charcoal, desalted and purified with ion exchangers in H--and OH--form, and fractionated using an "ODS" column for fractionation to obtain a high-purity maltotetraosyl-sucrose solution. To the solution was added 50 units/g dry matter of a thermostable non-reducing saccharide-forming enzyme from a strain of the genus **Sulfolobus** obtained by the method in Example for Reference 3, enzymatically reacted at 65.degree. C. for 20 hours, and heated to inactivate the remaining enzyme. The reaction mixture was in a usual manner decolored with an activated charcoal, desalted and purified with ion exchangers in H--and OH--form, concentrated, and spray dried to obtain a powder containing maltooligosylturanose and maltooligosylpalatinose in a yield of about 85%. The powder with non-crystallinity contained about 55% maltotetraosylturanose and maltotetraosylpalatinose and about 18% maltotetraosylsucrose. Because the

powder has a satisfactory sweetness, it can be arbitrarily used in food products, cosmetics, and pharmaceuticals.

DETL:
TABLE 2 ______________________________________ Amount of Reaction enzyme temperature Reaction Microorganism (unit/g) (.degree.C.) pH Yield* Yield** ______________________________________ Arthrobacter sp. 30 40 6.5 27.3 36.2 Q36 (FERM BP-4316) Rhizobium sp. 30 40 6.5 23.6 32.4 M-11 (FERM BP-4130) **Sulfolobus** 30 60 5.5 21.7 30.9 **acidocaldarius** (ATCC 33909) ______________________________________ Note : In the table, the symbols "yield*" and "Yield**" mean "the yield (%) of maltotetraosylturanose" and "the yield (%) of maltotetraosylpalatinose plus maltotetraosyltrehalulose".

CLPR:
5. The saccharide composition of claim 4, wherein said microorganism is one selected from the group consisting of those of the genera Rhizobium, Arthrobacter, and **Sulfolobus**.

USPT

US-CL-CURRENT: 530/412,530/414 ,530/422

US-PAT-NO: 5861295
DOCUMENT-IDENTIFIER: US 5861295 A
TITLE: Nucleic acid-free thermostable enzymes and methods of production thereof
DATE-ISSUED: January 19, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Goldstein; Adam S.	New Market	MD	N/A	N/A
Hughes, Jr.; A. John	Germantown	MD	N/A	N/A

US-CL-CURRENT: 435/194,530/412 ,530/414 ,530/422
ABSTRACT:
The present invention provides thermostable enzymes, such as DNA polymerases and restriction endonucleases, that are substantially free from contamination with nucleic acids. The invention also provides methods for the production of these enzymes, and kits comprising these enzymes which may be used in amplifying or sequencing nucleic acid molecules, including through use of the polymerase chain reaction (PCR).
16 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

BSPR:
These disruption approaches have several advantages, including their ability to rapidly and completely (in the case of physical methods) disrupt the bacterial cell such that the release of intracellular proteins is maximized. In fact, these approaches have been used in the initial steps of processes for the purification of a variety of bacterial cytosolic enzymes, including natural and recombinant proteins from mesophilic organisms such as Escherichia coli, Bacillus subtilis and Staphylococcus aureus (Laurent, S. J., and Vannier, F. S., Biochimie 59:747-750 (1977); Cull, M., and McHenry, C. S., Meth. Enzymol. 182:147-153 (1990); Hughes, A. J., Jr., et al., J. Cell. Biochem. Suppl. 0 16(Part B):84 (1992); Ausubel, F. M., et al., in: Current Protocols in Molecular Biology, New York: John Wiley & Sons, pp. 4.4.1-4.4.7 (1993)), as well as phosphatases, restriction enzymes, DNA or RNA polymerases and other proteins from thermophilic bacteria and archaea such as Thermus aquaticus, Thermus thermophilus, Thermus flavis, Thermus caldophilus, Thermotoga maritima, and **Sulfolobus acidocaldarius** (Shinomiya, T., et al., J. Biochem. 92(6):1823-1832 (1982); Elie, C., et al., Biochim. Biophys. Acta 951(2-3):261-267 (1988); Palm, P., et al, Nucl. Acids Res. 21(21):4904-4908 (1993); Park, J. H., et al., Eur. J. Biochem. 214(1):135-140 (1993); Harrell, R. A., and Hand, R. P., PCR Meth. Appl. 3(6):372-375 (1994); Meyer, W., et al., Arch. Biochem. Biophys. 319(1):149-156 (1995)).

DEPR:
Thermostable enzymes (e.g., DNA polymerases or restriction enzymes) may be prepared according to the methods of the present invention from a variety of thermophilic bacteria that are available commercially (for example, from American Type Culture Collection, Rockville, Md.). Suitable for use as sources of thermostable enzymes are the thermophilic bacteria Thermus aquaticus, Thermus thermophilus, Thermococcus litoralis, Pyrococcus furiosus, Pyrococcus woosii and other species of the Pyrococcus genus, Bacillus stearothermophilus, **Sulfolobus acidocaldarius,** Thermoplasma acidophilum, Thermus flavus, Thermus ruber, Thermus brockianus, Thermotoga neapolitana, Thermotoga maritima and other species of the Thermotoga genus, and Methanobacterium thermoautotrophicum, and mutants of each of these species. It will be understood by one of ordinary skill in the art, however, that any thermophilic microorganism may be used as a source for preparation of thermostable enzymes according to the methods of the present invention. Bacterial cells may be grown according to standard microbiological techniques, using culture media and incubation conditions suitable for growing active cultures of the particular species that are well-known to one of ordinary skill in the art (see, e.g., Brock, T. D., and Freeze, H., J. Bacteriol. 98(1):289-297 (1969); Oshima, T.,

and Imahori, K., Int. J. Syst. Bacteriol. 24(1):102-112 (1974)).

DEPR:
Following their purification, the substantially DNA-free thermostable enzymes may be stored until use in a buffered solution at temperatures of about -80.degree. to 25.degree. C., most preferably at -80.degree. to 4.degree. C., or in lyophilized form. Alternatively, the enzymes may be stabilized by drying in the presence of a sugar such as **trehalose** (U.S. Pat. Nos. 5,098,893 and 4,824,938) or acacia gum, pectin, carboxymethylcellulose, carboxymethylhydroxyethylcellulose, guar, carboxy guar, carboxymethylhydroxypropyl guar, laminaran, chitin, alginates or carrageenan. In addition, the enzymes provided by the present invention may be directly formulated into compositions to be used in techniques requiring the use of thermostable enzymes, such as compositions for nucleic acid sequencing or amplification in the case of thermostable DNA polymerases such as Taq, Tne, or Tma DNA polymerases, or mutants, derivatives or fragments thereof. These formulations may be concentrated solutions of the enzymes, or solutions of the enzymes at working concentrations which may comprise additional components and which may be prepared as described in co-pending U.S. patent application Ser. No. 08/689,815, by Ayoub Rashtchian and Joseph Solus, entitled "Stable Compositions for Nucleic Acid Sequencing and Amplification," filed Aug. 14, 1996, which is incorporated by reference herein in its entirety.

ORPL:
Meyer, W., et al., "Purification, Cloning, and Sequencing of Archaebacterial Pyrophosphatase from the Extreme Thermoacidophile **Sulfolobus acidocaldarius**," Arch. Biochem. Biophys. 319(1):149-156 (1995).

USPT

US-CL-CURRENT: 435/100,435/195 ,435/200 ,435/201 ,435/253.3 ,435/822 ,530/350 ,530/825

US-PAT-NO: 5856146
DOCUMENT-IDENTIFIER: US 5856146 A
TITLE: Recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharide
DATE-ISSUED: January 5, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/100 ,435/195 ,435/200 ,435/201 ,435/253.3 ,435/822 ,530/350 ,530/825
ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of **trehalose** on an industial scale and in a satisfactorily-high yield.
6 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

TTL:
Recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharide

ABPL:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of **trehalose** on an industial scale and in a satisfactorily-high yield.

BSPR:
The present invention relates to a recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:
Trehalose is a disaccharide which consists of 2 glucose molecules that are linked together with their reducing groups, and, in nature, it is present in fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, **trehalose** does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can advantageously sweeten food products without fear of causing unsatisfactory coloration and deterioration. **Trehalose**, however, could not have been readily prepared in a desired amount by conventional production methods, so that it has not widely been used for sweetening food products.

BSPR:
Conventional production methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other using a multi-enzymatic system where several enzymes are allowed to act on saccharides. The former, as

disclosed in Japanese Patent Laid-Open No.154,485/75, is a method which comprises growing microorganisms such as bacteria and yeasts in nutrient culture media, and collecting **trehalose** mainly from the proliferated cells. The latter, as disclosed in Japanese Patent Laid-Open No.216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and **trehalose**-phosphorylases to act on maltose, and recovering the formed **trehalose** from the reaction system. The former facilitates the growth of microorganisms, but has a demerit that the content in the microorganisms is at most 15 w/w %, on a dry solid basis (d.s.b.). Although the latter can readily separate **trehalose,** it is theoretically difficult to increase the **trehalose** yield by allowing such phosphorylases to act on substrates at a considerably-high concentration because the enzymatic reaction in itself is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

BSPR:
In view of the foregoing, the present inventors energetically screened enzymes which form saccharides having a **trehalose** structure from amylaceous saccharides, and have found that microorganisms such as those of the genera Rhizobium and Arthrobacter produce an absolutely novel enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. They disclosed such an enzyme in Japanese Patent Application No.349,216/93. At almost the same time, they also found that these non-reducing saccharides are nearly quantitatively hydrolyzed into **trehalose** and glucose and/or maltooligosaccharides by other enzymes produced from the same microorganisms of the genera Rhizobium and Arthrobacter.

BSPR:
It was found that the enzymes produced from the aforesaid microorganisms have an optimum temperature of about 40.degree. C., and have some difficulties in their thermostability when actually used to produce **trehalose**. It is recognized in this field that a recommended temperature in the saccharification reaction of starch or amylaceous saccharides is one which exceeds 55.degree. C. because bacterial contamination will occur at a temperature of 55.degree. C. or lower and decreasing the pH of the reaction mixtures and inactivating the enzymes used. Thus, a relatively-large amount of substrates remains intact. When using enzymes with poor thermostability, great care should be taken to control the pH, and, when the pH level drops to an extremely low level, alkalis should be added to reaction mixtures to increase the pH level as quickly as possible.

BSPR:
In view of the foregoing, the present inventors screened thermostable enzymes with a satisfactory activity and have found that enzymes produced from microorganisms of the genus **Sulfolobus** including **Sulfolobus acidocaldarius** (ATCC 33909) are not substantially inactivated even when incubated at a temperature exceeding 55.degree. C., and they efficiently release **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. These microorganisms, however, are not sufficient in enzyme productivity, and this requires a relatively-large scale culture to industrially produce **trehalose** from those non-reducing saccharides.

BSPR:
It is an object of the present invention to provide a recombinant thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 by using recombinant DNA technology.

BSPR:
It is another object of the present invention to provide an enzymatic conversion method for releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

BSPR:

The sixth object of the present invention is attained by an enzymatic conversion method of non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, which includes a step of allowing the recombinant thermostable enzyme to act on the non-reducing saccharides to release **trehalose**.

BSPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3;

DRPR:
FIG. 1 is a figure of the optimum temperature of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 2 is a figure of the optimum pH of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 3 is a figure of the thermostability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DRPR:
FIG. 4 is a figure of the pH stability of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
The recombinant thermostable enzyme according to the present invention releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 without substantial inactivation even when allowed to react at a temperature exceeding 55.degree. C.

DEPR:
According to the present invention, the enzymatic conversion method converts non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 into saccharide compositions containing **trehalose** and glucose and/or maltooligosaccharides.

DEPR:
The present invention has been made based on the finding of a novel enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. Such an enzyme is obtainable from cultures of microorganisms of the species **Sulfolobus acidocaldarius** (ATCC 33909). The present inventors isolated such an enzyme by using in combination various purification methods comprising column chromatography as a main technique, studied their properties and features, and revealed that the enzyme is a polypeptide with the following physicochemical properties:

DEPR:
The followings are the explanations of the experiments conducted to reveal the physicochemical properties of a thermostable enzyme produced from **Sulfolobus acidocaldarius** (ATCC 33909):

DEPR:
Into 500-ml flasks were poured 100 ml aliquots of a liquid culture medium containing 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min. After cooling the flasks a seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each liquid culture medium in each flask, followed by the incubation at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was poured into a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the first seed culture into the sterilized liquid

culture medium in the fermenter, and culturing the microorganisms at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was poured into a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and adjusted to a pH 3.0, followed by inoculating one v/v % of the second seed culture into the sterilized liquid culture medium, and culturing the microorganisms at 75.degree. C. for 42 hours under an aeration condition of 100 L/min.

DEPR:
The results in Table 1 show that the purified enzyme nearly quantitatively releases **trehalose** and glucose or maltooligosaccharides from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, but does not substantially act on maltooligosaccharides having a degree of glucose polymerization of at least 3. These facts indicate that the purified enzyme specifically acts on non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, and specifically hydrolyzes the glycosidic linkages between **trehalose**- and glycosyl-residues. Such an enzyme has not been reported and it can be hypothesized to have a novel enzymatic pathway.

DEPR:
A chromosomal DNA of **Sulfolobus acidocaldarius** (ATCC 33909) was screened by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequences in SEQ ID NOs:3 and 4, and this yielded a DNA fragment having a base sequence (SEQ ID NO:2) from the 5'-terminus consisting of about 1,700 base pairs in SEQ ID NO:2. The base sequence the thermostable enzyme was decoded and it was revealed that it consists of 556 amino acids and has a partial amino acid sequence from the N-terminal in SEQ ID NO:1.

DEPR:
To 500-ml flasks were placed about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water, and the flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled, and adjusted to a pH 3.0 by the addition of sulfate. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into each flask, incubated at 75.degree. C. for 24 hours under a rotary shaking condition of 130 rpm to obtain a seed culture. About 5 L of a fresh preparation of the same liquid nutrient culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to a pH 3.0, and inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min.

DEPR:
As a control, a seed culture of Escherichia coli XLI-Blue strain or **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated into a fresh preparation of the same liquid culture medium but free of ampicillin. In the case of culturing **Sulfolobus acidocaldarius** (ATCC 33909), it was cultured and treated similarly as above except that the initial pH of the nutrient culture medium and the culturing temperature were respectively set to 3.0 and 75.degree. C. Assaying the resultant enzymatic activity, one L culture of **Sulfolobus acidocaldarius** (ATCC 33909) yielded about 2 units of the thermostable enzyme, and the yield was significantly lower than that of transformant SU18. Escherichia coli XLI-Blue strain used as a host did not form the thermostable enzyme.

DEPR:
Thereafter, the recombinant thermostable enzyme produced by the transformant SU18 was purified similarly as in Experiments 1 and 2 and examined for properties and features and revealing that it has substantially the same physicochemical properties of the thermostable enzyme from **Sulfolobus acidocaldarius** (ATCC 33909) because (i) the recombinant thermostable enzyme has a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and an isoelectric point of about 5.6-6.6 on isoelectrophoresis, and (ii) it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These results indicate that the present

thermostable enzyme can be prepared by the recombinant DNA technology with a significantly improved yield.

DEPR:
Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of about 1,700 base pairs in SEQ ID NO:5. An amino acid sequence that could be estimated from the base sequence was in SEQ ID NO:5, and it was compared with the partial amino acid sequences in SEQ ID NOs:3 and 4, and revealing that the partial amino acid sequence in SEQ ID NO:3 corresponded to that positioning from 1 to 30 in SEQ ID NO:5, and that in SEQ ID NO:4 corresponded to that positioning from 301 to 319 in SEQ ID NO:5. These results indicate that the present recombinant thermostable enzyme has the amino acid sequence from the N-terminal in SEQ ID NO:1, and, in the case of the DNA derived from **Sulfolobus acidocaldarius** (ATCC 33909), the amino acid sequence is encoded by the base sequence from the 5'-terminus in SEQ ID NO:2.

DEPR:
As is explained in the above, the thermostable enzyme, which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, was found as a result of the present inventors' long-term research. The thermostable enzyme has distinct physicochemical properties from those of other conventional enzymes. The present invention is to produce the thermostable enzyme by using the recombinant DNA technology. The present recombinant thermostable enzyme, its preparation and uses will be explained in detail with reference to the later described Examples.

DEPR:
The recombinant thermostable enzyme as referred to in the present invention means thermostable enzymes in general which are preparable by recombinant DNA technology and capable of releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. Generally, the recombinant thermostable enzyme according to the present invention has a known amino acid sequence, and, as an example, the amino acid sequence from the N-terminal as shown in SEQ ID NO:1, and homologous ones to it can be mentioned. Variants having amino acid sequences homologous to the one in SEQ ID NO:1 can be obtained by replacing one or more bases in SEQ ID NO:1 with other bases without substantially alternating the inherent physicochemical properties. Although even when used the same DNA depending on the hosts into which the DNA is introduced, the ingredients and components of nutrient culture media for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have the inherent physicochemical properties but defect one or more amino acids in SEQ ID NO:1, or those which have one or more amino acids added newly to the N-terminal after the DNA expression as the result of the modification of intracellular enzymes of the hosts. Such variants can be used in the present invention as long as they have the desired physicochemical properties.

DEPR:
The DNA usable in the present invention includes those which are derived from natural resources and those which are artificially synthesized as long as they have the aforesaid base sequences. The natural resources for the DNA according to the present invention are, for example, microorganisms of the genus **Sulfolobus such as Sulfolobus acidocaldarius** (ATCC 33909), and from which genes containing the present DNA can be obtained. The aforementioned microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or .beta.-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, when the cells are treated with an ultrasonic disintegrator, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or by freezing and thawing. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment generally used in this field. To artificially synthesize the present DNA, it can be chemically synthesized by using the base sequence in SEQ ID NO:2, or can

be obtained in a plasmid form by inserting a DNA, which encodes the amino acid sequence in SEQ ID NO:1, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the objective DNA from the cells.

DEPR:
The recombinant DNA thus obtained can be introduced into appropriate host microorganisms including Escherichia coli and those of the genus Bacillus as well as actinomyces and yeasts. In the case of using Escherichia coli as a host, the DNA can be introduced thereinto by culturing the host in the presence of the recombinant DNA and calcium ion, while in the case of using a microorganism of the genus Bacillus as a host the competent cell method and the colony hybridization method can be used. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing reducing amylaceous saccharides having a degree of glucose polymerization of at least 3, and selecting the objective transformants which release **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

DEPR:
The transformants thus obtained intra- and extra-cellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid culture media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with small amounts of amino acids and vitamins can be used in the invention. Examples of the carbon sources are saccharides such as unprocessed starch, starch hydrolysate, glucose, fructose, sucrose and **trehalose**. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia and salts thereof, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor, and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 20.degree.14 65.degree. C. and a pH of 2-9 for about 1-6 days under aerobic conditions by the aeration-agitation method. Such cultures can be used intact as a crude enzyme, and, usually, cells in the cultures may be disrupted prior to use with ultrasonic and/or cell-wall lysis enzymes, followed by separating the thermostable enzyme from intact cells and cell debris by filtration and/or centrifugation and purifying the enzyme. The methods to purify the enzyme include conventional ones in general. From cultures intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separatory sedimentation, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

DEPR:
As is described above, the recombinant thermostable enzyme according to the present invention has a specific feature of releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 even when allowed to act on them at a temperature exceeding 55.degree. C. The **trehalose** thus obtained has a satisfactorily-mild and high-quality sweetness as well as an adequate viscosity and moisture-retaining ability, and, as a great advantageous feature, they can sweeten food products without fear of causing unsatisfactory coloration and deterioration because they have no reducing residue within their molecules. With these features a variety of amylaceous saccharides, which have been put aside because of their reducibilities, can be converted into saccharides which have a satisfactory handleability, usefulness, and no substantial reducibility or extremely-reduced reducibility.

DEPR:
Explaining now the conversion method in more detail, non-reducing saccharides having a **trehalose** structure and a degree of glucose polymerization of at least 3 such as .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose. These non-reducing saccharides can be

obtained by allowing a non-reducing saccharide-forming enzyme as disclosed in Japanese Patent Application No.349,216/93, applied by the present applicant and Japanese Patent Application Serial No.10046601, titled "Thermostable non-reducing saccharide-forming enzyme, its preparation and uses", applied by the same applicant on Jun. 24, 1994, to act on reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 which are prepared by treating starch or amylaceous saccharides such as amylopectin and amylose with acids and/or amylases. These reducing saccharides usable as a substrate for the non-reducing saccharide-forming enzyme usually contain one or more maltooligosaccharides having a degree of glucose polymerization of at least 3, for example, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. As is described in "Handbook of Amylases and Related Enzymes", 1st edition (1988), edited by The Amylase Research Society of Japan, published by Pergamon Press plc, Oxford, England, .alpha.-amylase, maltotetraose-forming amylase, maltopentaose-forming amylase and maltohexaose-forming amylase are especially useful to prepare the reducing amylaceous saccharides used in the present invention, and, the use of any one of these amylases facilitates the production of mixtures of amylaceous saccharides rich in reducing amylaceous saccharides having a degree of glucose polymerization of at least 3 in a considerably-high yield. If necessary, the combination use of the amylases and starch debranching enzymes such as pullulanase and isoamylase can increase the yield of the reducing amylaceous saccharides used as the substrate for the present recombinant thermostable enzyme. Non-reducing saccharides can be obtained in a desired amount by incorporating such a non-reducing saccharide-forming enzyme in aqueous solutions containing one or more reducing amylaceous saccharides up to 50 w/w %, and, usually, incubating the mixture solution at a temperature of 40.degree.-85.degree. C. and a pH of about 4-8 until the non-reducing saccharides are produced.

DEPR:
In the enzymatic conversion method according to the present invention, the present recombinant thermostable enzyme is generally allowed to coexist in an aqueous solution containing one or more of the above non-reducing saccharides as a substrate, followed by the enzymatic reaction at a prescribed temperature and pH until a desired amount of **trehalose** is formed. Although the enzymatic reaction proceeds even at a concentration of about 0.1 w/w %, d.s.b., of a substrate, a concentration of 2 w/w % or higher, d.s.b., preferably, in the range of 5-50 w/w %, d.s.b., of a substrate can be satisfactorily used when used the present conversion method in an industrial-scale production. The temperature and pH used in the enzymatic reaction are set to within a range which does not inactivate the recombinant thermostable enzyme and allows the enzyme to effectively act on substrates, i.e. a temperature of higher than 55.degree. C. but not higher than 85.degree. C., preferably, a temperature in the range of about 56.degree.-70.degree. C., and a pH of 4-7, preferably, a pH in the range of about 5-6. The amount and reaction time suitable for the present recombinant thermostable enzyme are chosen depending on the enzymatic reaction condition. Thus, the present recombinant thermostable enzyme effectively converts non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 into **trehalose** and glucose and/or maltooligosaccharides, e.g. the conversion rate increases up to about 99% when the enzyme acts on .alpha.-maltotriosyltrehalose. When either of the amylases is allowed to act on starch hydrolysates in combination with the non-reducing saccharide-forming enzyme and the present thermostable enzyme, non-reducing saccharides are formed along with **trehalose** and glucose and/or maltooligosaccharides. Thus, saccharide compositions rich in **trehalose** are efficiently formed in a relatively-high yield.

DEPR:
The **trehalose** and compositions containing it have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
The purified enzyme was assayed for properties and features by the method in Experiment 2 and revealing that it had a molecular weight of about 54,000-64,000 daltons on SDS-PAGE and a pI of about 5.6-6.6 on

isoelectrophoresis, and was not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min. These physicochemical properties were substantially the same as those of the thermostable enzyme from a donor microorganism of **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
Conversion into syrupy product containing **trehalose**

DEPR:
To 500-ml flasks were added about 100 ml aliquots of a liquid culture medium consisting of 0.1 w/v % polypeptone, 0.1 w/v % yeast extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium dihydrogen phosphate, 0.02 w/v % magnesium sulfate heptahydrate, 0.02 w/v % potassium chloride, and water. The flasks were sterilized by autoclaving at 120.degree. C. for 20 min, cooled and the pH of the contents was adjusted to 3.0 by the addition of sulfuric acid. A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated to the flasks, and cultured at 75.degree. C. for 24 hours under a rotatory shaking condition of 130 rpm to obtain a first seed culture. About 5 L of a fresh preparation of the same liquid culture medium was placed in a 10-L fermenter, sterilized similarly as above, cooled to 75.degree. C., adjusted to pH 3.0, inoculated with one v/v % of the seed culture, followed by the incubation at 75.degree. C. for 24 hours under an aeration condition of 500 ml/min to obtain a second seed culture. Thereafter, about 250 L of a fresh preparation of the same liquid culture medium was introduced into a 300-L fermenter, sterilized similarly as above, cooled to 75.degree. C., and cultured under aeration and agitation conditions of 100 L/min for 42 hours.

DEPR:
Fractions with an enzymatic activity eluted at about 0.8M ammonium sulfate were collected, pooled, dialyzed for 16 hours against 10 mM Tris-HCl buffer (pH 8.5) containing 0.2M sodium chloride, and centrifuged at 10,000 rpm for 30 min to remove insoluble substances. The resultant supernatant was fed to a column packed with about 350 ml of "TOYOPEARL.RTM. HW-55", a gel for gel chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 0.2M sodium chloride. Fractions with an enzymatic activity were collected from the eluate, pooled, and dialyzed against 10 mM Tris-HCl buffer (pH 8.5) for 16 hours. The dialyzed solution was centrifuged to remove insoluble substances, and the supernatant was subjected to hydrophobic column chromatography using "MONO Q", a gel for ion-exchange chromatography commercialized by Pharmacia LKB Uppsala, Sweden, followed by feeding to the column with a linear gradient buffer ranging from 0M to 0.2M of sodium chloride in 10 mM Tris-HCl buffer (pH 8.5). The fractions eluted at about 0.1M sodium chloride were collected and pooled for the production of **trehalose**. The purified non-reducing saccharide-forming enzyme thus obtained had a specific activity of about 81 units/mg protein, and the yield was about 0.24 units per one L of the culture.

DEPR:
Conversion into syrupy product containing **trehalose**

DEPR:
The syrup had a relatively-low DE (dextrose equivalent) and contained 71.0 w/w % **trehalose,** 2.9 w/w % glucosyltrehalose, 1.0 w/w % maltosyltrehalose, 4.9 w/w % glucose, 10.5 w/w % maltose, 8.2 w/w % maltotriose and 1.5 w/w % maltotetraose and higher maltooligosaccharides, d.s.b. The product, having a mild and moderate sweetness as well as an adequate viscosity and moisture-retaining ability, can be satisfactorily used in compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
Conversion into powdery product containing **trehalose**

DEPR:
A syrupy product in Example B-1 was column chromatographed using a strong-acid cation exchange resin to increase the **trehalose** content. The procedures were

as follows: Four jacketed-stainless steel columns, 5.4 cm in diameter and 5 m in length each, were packed to homogeneity with "XT-1016 (Na.sup.+ -form)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, which had been previously suspended in water, and cascaded in series to give a total column length of 20 m. The columns were fed with the syrupy product, adequately diluted with water, in a volume of about 5 v/v % to the resin and at an inner column temperature of 55.degree. C., and fed with 55.degree. C. hot water at an SV (space velocity) 0.13 to elute saccharide components. Fractions rich in **trehalose** were collected, pooled, concentrated, dried in vacuo and pulverized to obtain a powdery product containing about 97 w/w % **trehalose** in a yield of about 55 w/w % to the material, d.s.b.

DEPR:
A fraction rich in **trehalose** obtained by the method in Example B-2 was concentrated into an about 75 w/w % solution which was then transferred to a crystallizer, and crystallized under gently stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was sprayed downward from a nozzle equipped on the upper part of a spraying tower at a pressure of about 150 kg/cm.sup.2 while an about 85.degree. C. hot air was blowing downward from the upper part of the spraying tower, and the formed crystalline powder was collected on a wire-netting conveyer provided on the basement of the drying tower and gradually conveyed out of the spraying tower while hot air at about 45.degree. C. was blowing to the crystalline powder from under the conveyer. The crystalline powder thus obtained was transferred to an ageing tower and aged for 10 hours in a stream of about 40.degree. C. hot air to complete the crystallization and drying. Thus, a powdery hydrous crystalline **trehalose** was obtained in a yield of about 90 w/w % to the material, d.s.b.

DEPR:
Conversion into powdery product containing crystalline **trehalose**

DEPR:
Tapioca starch was dissolved in water into a 36 w/w % suspension which was then admixed with 0.1 w/w % calcium carbonate. The mixture was adjusted to pH 6.0, admixed with 0.2 w/w % of "TERMAMYL 60L", an .alpha.-amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, to starch, d.s.b., and enzymatically reacted at 95.degree. C. for 15 min to gelatinize and liquefy the starch. The mixture was autoclaved at 120.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 58.degree. C., adjusted to pH 5.2, admixed with 2,000 units/g starch, d.s.b., of an isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 2.5 units/g starch, d.s.b., of a thermostable enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 72 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, cooled to 50.degree. C., admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered, decolored in a usual manner with an activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 75.5 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was concentrated into an about 84 w/w % solution which was then transferred to a crystallizer, admixed with about 2 w/w %, d.s.b., of hydrous crystalline **trehalose** as a seed crystal, and crystallized under gentle stirring conditions to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b. The massecuite was distributed to plastic plain vessels, allowed to stand at ambient temperature for 3 days to solidify and age the contents. Thereafter, the formed blocks were removed from the vessels, powdered by a pulverizer to obtain a solid product containing hydrous crystalline **trehalose** in a yield of about 90 w/w % to the material starch, d.s.b.

DEPR:
Conversion into powder product containing crystalline **trehalose**

DEPR:
Potato starch was suspended in water into a 6 w/w % suspension which was then admixed with 0.01 w/w % "NEO-SPITASE", an .alpha.-amylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, adjusted to pH 6.2, and subjected to an enzymatic reaction at a temperature of 85.degree.-90.degree. C. for one hour to gelatinize and liquefy the starch. The mixture was heated at 120.degree. C. for 10 min to inactivate the remaining enzyme, cooled to 60.degree. C., adjusted to pH 5.5, admixed with 500 units/g starch, d.s.b., of "PROMOZYME 200L", a pullulanase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, 3.0 units/g starch, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a), 5.0 units/g starch, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours. The reaction mixture thus obtained was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered, decolored a in usual manner with activated charcoal, desalted and purified with ion exchangers, and concentrated into an about 60 w/w % syrup to obtain a syrupy product containing about 79.3 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was column chromatographed similarly as in Example B-2 except that "C6000", commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used a strong-acid cation exchange resin in Na.sup.+ -form, followed by collecting a fraction containing about 95 w/w % **trehalose,** d.s.b. The fraction was concentrated up to about 75 w/w % and crystallized similarly as in Example B-4 to obtain a massecuite in the from of a block which was then pulverized to obtain a powdery product containing hydrous crystalline **trehalose** in a yield of about 70 w/w % to the material starch, d.s.b.

DEPR:
Conversion into powdery product containing anhydrous crystalline **trehalose**

DEPR:
One part by weight of "EX-I", an amylose product commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was dissolved by heating in 15 parts by weight of water, and the solution was adjusted to 65.degree. C. and pH 5.5, admixed with 2.0 units/g amylose, d.s.b., of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example B-1 (a) and 6.0 units/g amylose, d.s.b., of a recombinant thermostable enzyme obtained by the method in Example A-2, and subjected to an enzymatic reaction for 48 hours. The reaction mixture was incubated at 97.degree. C. for 30 min to inactivate the remaining enzyme, adjusted to 50.degree. C. and pH 5.0, admixed with 10 units/g amylose, d.s.b., "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and incubated for another 40 hours. The newly formed reaction mixture was heated at 95.degree. C. for 10 min to inactivate the remaining enzyme, cooled, filtered in a usual manner, decolored with an activated charcoal, deionized and purified with an ion exchanger, and concentrated up to give a concentration of about 60 w/w % to obtain a syrupy product containing 82.2 w/w % **trehalose,** d.s.b.

DEPR:
The syrupy product was similarly as in Example B-5 subjected to column fractionation to obtain a fraction containing 98 w/w % **trehalose,** d.s.b., which was then concentrated by heating under reduced pressure up to give a concentration of about 85 w/w %. To the concentrate was added about 2 w/w % anhydrous crystalline **trehalose** as a seed, followed by mixing the resultant at 120.degree. C. for 5 min under stirring conditions. The resultant mixture was distributed to plastic plain vessels, and crystallized by drying in vacuo at 100.degree. C. Thereafter, products in the form of a block were removed from the vessels, pulverized with a cutter to obtain a solid product, which contained anhydrous crystalline **trehalose** and had a moisture content of about

0.3 w/w % and a crystallization percentage of about 70 w/w %, in a yield of about 70% to the material amylose, d.s.b.

DEPR:
Anhydrous crystalline **trehalose** absorbs moisture from anhydrous substances to convert into hydrous crystalline **trehalose,** and because of this the product rich in such anhydrous crystalline **trehalose** is useful as a desiccant to dehydrate compositions such as food products, cosmetics and pharmaceuticals, and their materials and intermediates. The product with a mild and high-quality sweetness can be arbitrarily incorporated into compositions in general such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, diluent and excipient.

DEPR:
As is described above, the present invention is based on the finding of a novel thermostable enzyme which releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3. The present invention provides a way to produce such a thermostable enzyme on an industrial scale and in a relatively-high efficient manner by recombinant DNA technology. The present conversion method using the recombinant thermostable enzyme readily converts non-reducing amylaceous saccharides, which have a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, into **trehalose** and glucose and/or maltooligosaccharides without fear of causing bacterial contamination. The **trehalose** has a mild and high-quality sweetness, and, because it has no reducing residue within the molecule, it can be advantageously incorporated into compositions in general such as food products, cosmetics and pharmaceuticals without fear of causing unsatisfactory coloration and deterioration. The present recombinant thermostable enzyme is one which has a revealed amino acid sequence, so that it can be used freely in the preparation of **trehalose** that is premised to be used in food products and pharmaceuticals.

DEPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3;

DETL:
TABLE 1

Saccharide in Elution time on HPLC Composition Substrate reaction mixture (min) (%)

.alpha.-Glucosyltrehalose **Trehalose** 27.4 7.2 Glucose 33.8 3.9 .alpha.-Glucosyltrehalose 23.3 88.9 .alpha.-Maltosyltrehalose **Trehalose** 27.4 40.2 Maltose 28.7 40.5 .alpha.-Maltosyltrehalose 21.6 19.3 .alpha.-Maltotriosyltrehalose **Trehalose** 27.4 41.1 Maltotriose 25.9 58.2 .alpha.-Maltotriosyltrehalose 19.7 0.7 .alpha.-Maltotetraosyltrehalose **Trehalose** 27.4 34.0 Maltotetraose 24.1 65.8 .alpha.-Maltotetraosyltrehalose 18.7 0.2 .alpha.-Maltopentaosyltrehalose **Trehalose** 27.4 29.1 Maltopentaose 22.6 70.6 .alpha.-Maltopentaosyltrehalose 17.8 0.3 Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

CLPR:
3. An enzymatic conversion method, which comprises a step of contacting the recombinant thermostable enzyme of claim 1 with a non-reducing saccharide, which non-reducing saccharide has a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, to produce **trehalose**.

CLPR:
6. The method of claim 3, wherein said non-reducing saccharide is a member selected from the group consisting of .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyl**-trehalose,** .alpha.-maltopentaosyltrehalose, and mixtures thereof.

CLPW:
Releasing **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3, but substantially not acting on maltooligosaccharides having a degree of glucose polymerization of at least,3;

USPT

US-CL-CURRENT: 536/123.1,536/124

US-PAT-NO: 5789392
DOCUMENT-IDENTIFIER: US 5789392 A
TITLE: Saccharide composition with reduced reducibility, and preparation and uses thereof
DATE-ISSUED: August 4, 1998
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/54,536/123.1 ,536/124
ABSTRACT:
A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction.
9 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

ABPL:
A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction.

BSPR:
The present invention relates to a saccharide composition with a reduced reducibility, and preparation and uses thereof, more particularly, to a saccharide composition with a educed reducibility which comprises a sugar alcohol and a non-reducing saccharide consisting of **trehalose** and/or a saccharide having a **trehalose** structure.

BSPR:
Trehalose or .alpha.,.alpha.**-trehalose,** a non-reducing saccharide consisting of two glucopyranoside residues, has been well known. As is described in Advances in Carbohydrate Chemistry, Vol.18, pp.201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), **trehalose** is widely distributed in microorganisms, mushrooms, insects, etc., though the content is relatively low. Since non-reducing saccharides including **trehalose** do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, such non-reducing saccharides have been expected to be used without fear of causing unsatisfiable browning and deterioration, and the preparation of which has been in great demand.

BSPR:
Examples of conventional preparations of **trehalose** include a method as disclosed in Japanese Patent Laid-Open No.154,485/75 wherein microorganisms are utilized, and a conversion method as proposed in Japanese Patent Laid-Open No.216,695/83 wherein maltose is converted into **trehalose** by the combination use of maltose- and **trehalose**-phosphorylases. The former is, however, not suitable for the industrial-scale preparation of **trehalose** because the content of **trehalose** present in microorganisms as a starting material is usually lower

than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the present specification, unless specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, the concentration of maltose as a substrate could not be set to a relatively-high concentration; (ii) Since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) It is substantially difficult to retain the reaction systems stably and to continue their enzymatic reactions smoothly. Therefore, they have not been established as an industrial-scale preparation.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No.88, pp.67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been recognized to be scientifically almost impossible." so that an enzymatic preparation of **trehalose** from starch as a material has been recognized to be scientifically difficult.

BSPR:
To overcome such conventional drawbacks, the present inventors had disclosed in Japanese Patent Application No.349,216/93 a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from one or more reducing partial starch hydrolysates with a glucose polymerization degree of 3 or more (throughout the present specification, the enzyme is designated as "non-reducing saccharide-forming enzyme"). By using the non-reducing saccharide-forming enzyme, they established the preparation of non-reducing saccharides, which have a glucose polymerization degree of 3 or more and a **trehalose** structure as an end unit, from reducing partial starch hydrolysates, as well as establishing saccharide compositions with a reduced reducibility and the preparation of **trehalose** from these non-reducing saccharides and saccharide compositions.

BSPR:
The present inventors also disclosed in Japanese Patent Application No.79,291/94 a novel **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and others in non-reducing saccharides having a glucose polymerization degree of 3 or more and a **trehalose** structure as an end unit (throughout the present specification, the enzyme is designated as "**trehalose**-releasing enzyme"), and established the preparation of **trehalose** in a relatively-high yield from reducing partial starch hydrolysates by the combination use of the aforesaid non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme. It was revealed that in the preparations of saccharide compositions having a **trehalose** structure and a glucose polymerization degree of 3 or more and those of non-reducing saccharides such as **trehalose,** intact reducing partial starch hydrolysates still remain in the final products, and reducing amylaceous saccharides such as glucose and maltose are newly formed. Required is to more reduce the reducibility of these saccharide compositions with a reduced reducibility which contain the aforesaid non-reducing saccharides and reducing saccharides.

BSPR:
The present invention is to provide saccharides having a **trehalose** structure or non-reducing saccharides having a **trehalose** structure as an end unit (hereinafter designated as ".alpha.-glycosyltrehalose" in the present specification), non-reducing saccharides wherein one or more glucose residues bind to the two glucopyranoside residues of **trehalose** (hereinafter designated as ".alpha.-glycosyl .alpha.-glycoside" in the present specification), and saccharides prepared by more reducing the reducibility of those with a relatively-low reducibility which contain reducing saccharides and non-reducing saccharides such as **trehalose**. The present invention is to also provide the preparation and uses of these saccharides.

BSPR:
As a result, they found that reducing amylaceous saccharides can be readily converted into their corresponding sugar alcohols by hydrogenating saccharide

compositions with a reduced reducibility comprising the reducing amylaceous saccharides and other non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure without fear of the affection of the non-reducing saccharides, and that the reducibility of the saccharide compositions as a material is lowered or even substantially diminished.

BSPR:
The present inventors studied on the preparation of the materials usable in the present invention, i.e. saccharide compositions with a relatively-low reducibility which comprise reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, and have found that compositions which are obtainable by allowing a non-reducing saccharide-forming enzyme together with or without a **trehalose**-releasing enzyme to act on reducing partial starch hydrolysates with a glucose polymerization degree of 3 or more can be arbitrarily used. More particularly, saccharide compositions with a low reducibility, which are obtainable by allowing starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on liquefied starch solutions when a non-reducing saccharide-forming enzyme is allowed to act on the solutions together with or without a **trehalose**-releasing enzyme, are satisfactorily used. Furthermore, the present inventors found that saccharide compositions with a low reducibility, which are obtainable by allowing a maltose-**trehalose** converting enzyme to act on amylaceous substances, as disclosed in Japanese Patent Application No.144,092/94, titled "Maltose-**trehalose** converting enzyme, and preparation and uses thereof", applied for by the present applicant on the day of Jun. 3, 1994, can be arbitrarily used in the present invention. Thus, they accomplished this invention and revealed that, in the process for preparing such saccharide compositions with a reduced reducibility wherein a non-reducing saccharide-forming enzyme is allowed to act on amylaceous solutions with a DE less than 15, saccharide compositions with a low reducibility, which contain non-reducing saccharides obtainable by allowing a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on the amylaceous solutions, can be suitably used as a material saccharide in the present invention because they have an extremely reduced molecular weight and viscosity and a satisfactory handleability without substantial increment of their initial reducibility than those prepared by the sole use of the non-reducing saccharide-forming enzyme. They also found that the **trehalose** content in the saccharide compositions with a relatively-low reducibility increases when glucoamylase acts on them. In addition, they found that, in the process for preparing **trehalose** by allowing a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme to act on liquefied starch solutions with a relatively-low DE, preferably, those with a DE less than 15, **trehalose** which is obtainable by using a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase can be suitably used as a material in the present invention because such **trehalose** is obtained in a relatively-high yield as compared with that obtained by the sole use of the non-reducing saccharide-forming enzyme. Furthermore, it was found that saccharide compositions of **trehalose** and maltose, which are obtainable by allowing a maltose-**trehalose** converting enzyme to act on maltose, can be arbitrarily used in the present invention. The resultant saccharide compositions with a low reducibility and rich in non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure can be readily hydrogenated. These saccharide compositions are substantially free of reducibility or they have a dextrose equivalent (DE) value less than 1, and have a satisfactory stability, handleability and wide applicability. Thus, they can be arbitrarily used in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DRPR:
FIG. 9 shows the elution patterns of a **trehalose**-releasing enzyme and a non-reducing saccharide-forming enzyme usable in the present invention on column chromatography using "TOYOPEARL".

DRPR:
FIG. 10 shows the influence of temperature on a **trehalose**-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 12 shows the influence of temperature on the stability of a

trehalose-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 13 shows the influence of pH on the stability of a trehalose-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 14 shows the influence of temperature on the activity of a trehalose-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 15 shows the influence of pH on the activity of a trehalose-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 16 shows the influence of temperature on the activity of a trehalose-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 17 shows the influence of pH on the activity of a trehalose-releasing enzyme derived from Arthrobacter sp. Q36.

DEPR:
The non-reducing saccharide-forming enzymes usable in the present invention include those which can form .alpha.-glycosyltrehalose from one or more reducing amylaceous partial starch hydrolysates selected from those with a glucose polymerization degree of 3 or more which are contained in liquefied starch solutions with a relatively-low DE. Examples of such enzymes are those derived from microorganisms of the genera Rhizobium, Arthrobacter, Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium, Mycobacterium and Terrabacter as disclosed in Japanese Patent Application No.349,216/93. If necessary, thermostable non-reducing saccharide-forming enzymes can be arbitrarily used in the present invention. For example, a thermostable non-reducing saccharide-forming enzyme derived from a microorganism of the genus Sulfolobus as disclosed in Japanese Patent Application No.166,011/94, titled "Thermostable non-reducing saccharide-forming enzyme, and its preparation and uses", applied for by the present applicant on the day of Jun. 24, 1994, can be arbitrarily used. Any enzyme, which specifically hydrolyzes the linkage between a trehalose moiety and others in .alpha.-glycosyltrehalose formed by allowing a non-reducing saccharide-forming enzyme to act on a liquefied starch solution, can be used as a trehalose-releasing enzyme in the present invention: For example, those derived from microorganisms of the genera Rhizobium, Arthrobacter, Brevibacterium and Micrococcus as disclosed in Japanese Patent Application No.79,291/94 can be arbitrarily used. If necessary, thermostable trehalose-releasing enzymes such as that derived from a microorganism of the genus Sulfolobus as disclosed in Japanese Patent Application No.166,126/94, applied for by the present applicant on the day of Jun. 25, 1994, can be arbitrarily used in the present invention.

DEPR:
Any enzyme can be arbitrarily used in the present invention as a maltose-trehalose converting enzyme as long as it forms trehalose: Examples of such an enzyme are those derived from microorganisms of the genera Pimerobacter, Pseudomonas and Thermus as disclosed in Japanese Patent Application No.144,092/94, titled "Maltose-trehalose converting enzyme, and preparation and uses thereof", applied for by the present applicant on the day of Jun. 3, 1994. The methods used for preparing the non-reducing saccharide-forming enzymes, trehalose-releasing enzymes, and maltose-trehalose converting enzymes in the present invention are those which comprise culturing microorganisms capable of forming such enzymes in nutrient culture media, and collecting the formed enzymes.

DEPR:
The trehalose-releasing enzymes usable in the present invention generally have the following physicochemical properties:

DEPR:
The activity of the trehalose-releasing enzymes is assayed as follows: One ml

of an enzyme solution is added to 4 ml of 1.25 w/v % maltotriosyl **trehalose** alias .alpha.-maltotetraosyl .alpha.-D-glucoside in 50 mM phosphate buffer (pH 7.0), and the mixture solution is incubated at 40.degree. C. for 30 min. To the resultant reaction mixture is added a copper solution for the Somogyi reaction to suspend the enzymatic reaction, followed by the determination of the reducing power on the Somogyi-Nelson's method. As a control, an enzyme solution, which was preheated at 100.degree. C. for 10 min to inactivate the enzyme, is assayed similarly as above. One unit activity of the enzyme is defined as the amount of enzyme which increases the reducing power of that of one .mu.mole of glucose per minute when assayed with the above-mentioned assay.

DEPR:
The maltose-**trehalose** releasing enzymes usable in the present invention have the following physicochemical properties:

DEPR:
The activity of the maltose-**trehalose** converting enzymes is assayed as follows: One ml of an enzyme solution is added to one ml of 20 w/v % maltose as a substrate in 10 mM phosphate buffer (pH 7.0), and the mixture solution is incubated at 25.degree. C. for 60 min, followed by heating the solution at 100.degree. C. for 10 min to suspend the enzymatic reaction. To the resultant reaction mixture is precisely diluted by 11-fold with 50 mM phosphate buffer (pH 7.5), and 0.4 ml of the diluted solution is admixed with 0.1 ml of an enzyme solution containing one unit/ml trehalase. The resultant solution is incubated at 45.degree. C. for 120 min, followed by determining the amount of glucose by the glucose-oxidase method. As a control, by using trehalase and an enzyme solution, which were preheated at 100.degree. C. for 10 min to inactivate the enzymes, the resultant enzyme solution is assayed similarly as above. With the above assay, the content of **trehalose,** formed by the maltose-**trehalose** converting enzyme, is determined based on the amount of the formed glucose, and one unit activity of the enzyme is defined as the amount of enzyme which forms one pmole of **trehalose** per minute.

DEPR:
The liquefied starch solutions thus obtained can be subjected to the action of a non-reducing saccharide-forming enzyme together with a starch debranching enzyme and/or a cyclomaltodextrin glucanotransferase, or to the action of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme together with a starch debranching enzyme and/or a cyclomaltodextrin glucanotransferase under the pH--and temperature-conditions which allow these enzymatic reactions to proceed, for example, a pH of 4-10, preferably, of 5-8, and a temperature of about 10.degree.-80.degree. C., preferably, of about 30.degree.-70.degree. C. The order of the enzymes to be added to the liquefied starch solutions is not specifically restricted to, for example, one or more of these enzymes can be first added to the solutions, then the remaining enzyme(s) is added to, or all the enzymes can be added to at the same time.

DEPR:
The amounts of the enzymes to be added can be chosen depending upon the enzymatic conditions and reaction times. Usually, they are chosen from (i) about 0.01-100 units/g substrate, d.s.b., in liquefied starch solutions for the enzymatic reaction of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme, (ii) about 1-10,000 units/g substrate, d.s.b., for starch debranching enzymes, and (iii) about 0.05-500 units/g substrate, d.s.b., for cyclomaltodextrin glucanotransferases. The resultant saccharide compositions with a reduced reducibility, which comprise non-reducing saccharides and reducing amylaceous saccharides, are prepared by subjecting liquefied starch solutions to the action of starch debranching enzymes and/or cyclomaltodextrin glucanotransferases and non-reducing saccharide-forming enzymes together with or without **trehalose**-releasing enzymes so that the saccharide compositions have characters that they contain **trehalose** in quantity or considerably-large amounts of relatively-low molecular weight .alpha.-glycosyltrehalose and/or .alpha.-glycosyl .alpha.-glycoside, and that they can be arbitrarily used as a material saccharide composition with a reduced reducibility in the present invention. The .alpha.-glycosyl .alpha.-glycoside is a name given to a compound such as .alpha.-D-oligoglycosyl .alpha.-D-oligoglucoside as disclosed in Japanese Patent Application No.54,377/94 applied for by the present applicant.

DEPR:
If necessary, the resultant saccharide compositions with a reduced reducibility, which contain non-reducing saccharides having a **trehalose** structure, can be further processed into the material saccharide composition with a reduced reducibility used in the present invention by hydrolyzing them with amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase or .alpha.-glucosidase to control their sweetness and/or lower their viscosity.

DEPR:
The saccharide compositions thus obtained can be arbitrarily used as a material saccharide composition in the present invention. For example, those rich in non-reducing saccharides consisting of **trehalose** and saccharides having a **trehalose** structure, i.e. those which contain 20% or more, preferably, 40% or more, more preferably, 60% or more of **trehalose,** d.s.b., and have a relatively-low DE, usually, a DE less than 70, preferably, a DE less than 50, more preferably, a DE less than 30, are satisfactorily used. Varying dependently on their compositions, the material saccharide compositions used in the present invention have features that they have a relatively-low DE regardless of their large amount of non-reducing reducing saccharides, relatively-low molecular weight substances to be tasted, and relatively-low viscosity, and that they facilitate the hydrogenation and the subsequent processes such as purification and concentration wherein the amount of hydrogen which is required for the hydrogenation is reduced by a large margin.

DEPR:
Any method for hydrogenating the resultant saccharide compositions with a reduced reducibility, which comprise reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, can be used in the present invention as long as it does not decompose the non-reducing saccharides but hydrogenates saccharides into sugar alcohols. For example, the material saccharide compositions are prepared into 30-70% aqueous solutions, transferred to an autoclave, mixed with about 8-10% Raney nickel as a catalyst, and heated up to a temperature of 90.degree.-150.degree. C. under stirring conditions to terminate the hydrogenation or, preferably, to partially hydrogenate the contents until they show a DE less than 0.5, followed by removing the Raney nickel. The resultant mixtures were decolored with an activated charcoal in usual manner, desalted with an ion-exchange resin, and concentrated into syrupy products. If necessary, the syrupy products are arbitrarily dried into powdery products, or crystallized into crystalline powders containing **trehalose** crystal. The resultant saccharide compositions with a reduced reducibility contain non-reducing saccharides, which consist of **trehalose** and/or non-reducing saccharides having a **trehalose** structure, such as .alpha.-glycosyltrehalose and .alpha.-glycosyl .alpha.-glycoside, and one or more sugar alcohols such as sorbitol, maltitol, maltotriitol, maltotetraiol and maltopentaitol.

DEPR:
The saccharide compositions according to the present inventions form relatively-low molecular weight non-reducing oligosaccharides and maltooligosaccharides when hydrolyzed with amylases such as .alpha.-amylase derived from pancreas. The oligosaccharides are readily hydrolyzed with .alpha.-glucosidase and enzymes in small intestines into glucose and **trehalose** which is then readily hydrolyzed with trehalase into glucose molecules. Thus, the present saccharide compositions are readily assimilated, absorbed and utilized by living bodies when orally administered. Furthermore, they are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
Fifty mg aliquots of non-reducing saccharide preparations P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for 6 hours. HPLC analysis only detected glucose and **trehalose** in all resultant mixtures. The percentages and the molecular ratios of glucose to **trehalose** in each saccharide are in Table 5.

DEPR:
As is evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one glucose molecule and one **trehalose** molecule; P II, hydrolyzed into 2 glucose molecules and one **trehalose** molecule; (iii) P III, hydrolyzed into 3 glucose molecules and one **trehalose** molecule; (iv) P IV, hydrolyzed into 4 glucose molecules and one **trehalose** molecule; and (v) P V, hydrolyzed into 5 glucose molecules and one **trehalose** molecule.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more glucose molecules bound to one **trehalose** molecule via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing saccharide which has a glucose polymerization degree of 3 (DP 3) and consists of one glucose molecule bound to one **trehalose** molecule; P II, a non-reducing saccharide which has DP 4 and consists of 2 glucose molecules bound to one **trehalose** molecule; P III, a non-reducing saccharide which has DP 5 and consists of 3 glucose molecules bound to one **trehalose** molecule; P IV, a non-reducing saccharide which has DP 6 and consists of 4 glucose molecules bound to one **trehalose** molecule; and P V, a non-reducing saccharide which has DP 7 and consists of 5 glucose molecules bound to one **trehalose** molecule. It was revealed that, when .beta.-amylase was act on these non-reducing saccharides similarly as in glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one maltose molecule and one P I molecule, one maltose molecule and one P II molecule, and 2 maltose molecules and one P I molecule.

DEPR:
Based on these results, it was concluded that the enzymatic reaction mechanism of the present non-reducing saccharide-forming enzymes is an intramolecular conversion reaction without changing the molecular weights of substrates, i.e. an intramolecular conversion reaction without changing the glucose polymerization degrees of substrates. It was also concluded that the non-reducing saccharides P I, P II, P III, P IV and P V were respectively .alpha.-glycosyltrehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.-**trehalose** residue) of .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose.

DEPR:
As is evident from the results in Tables 7 and 8, it was revealed that, similarly as in Experiment 6 wherein glucoamylase is used, the saccharide preparations P I, P II, P III, P IV and P V were hydrolyzed by the .alpha.-glucosidase and the rat intestinal acetone powder into glucose and **trehalose**.

DEPR:
To the resultant hydrolysate obtained by the .alpha.-glucosidase or the rat intestinal acetone powder was added one unit trehalase derived from pig kidney, a product of Sigma Chemical Company, St., Louis, USA, and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC revealing that **trehalose**, formed from the saccharide preparations P I, P II, P III, P IV and P V, was hydrolyzed by trehalase into glucose molecules.

DEPR:
Based o n these results, it was concluded that the non-reducing saccharide-forming enzyme used in the present invention is an enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates into a non-r educing end unit, i.e. a **trehalose** residue or a **trehalose** structure.

DEPR:
The followings are the explanations of **trehalose**-releasing enzymes from Rhizobium sp. M-11 and Arthrobacter sp. Q36, as well as from known microorganisms:

DEPR:
The activities of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme accumulated in the culture were respectively about 1.5 units/ml and about 2 units/ml. A portion of the culture was centrifuged into cells and a culture supernatant, and the cells were suspended in 50 mM phosphate buffer (pH 7.0) to give the same volume of the portion, followed by assaying the enzyme activities of the cell suspension and the culture supernatant. The activities of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme in the cell suspension were respectively about 0.6 units/ml and about 0.8 units/ml, and the culture supernatant contained about 0.9 units/ml of the non-reducing saccharide-forming enzyme and about 1.2 units/ml of the **trehalose**-releasing enzyme.

DEPR:
The objective non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme were adsorbed on the ion-exchanger, and eluted separately from the column with a fresh preparation of the same phosphate buffer supplemented with salt at different salt concentrations. The elution pattern from the column or the column chromatogram is in FIG. 9. The non-reducing saccharide-forming enzyme was eluted from the column at a salt concentration of about 0.2 M, while the **trehalose**-releasing enzyme was eluted from the column at a salt concentration of about 0.3 M. The fractions containing either of the objective enzymes were separately pooled and purified as follows:

DEPR:
Fractions with a **trehalose**-releasing enzyme activity eluted from the column of "DEAE-TOYOPEARL.RTM." were pooled and treated similarly as in the purification steps used in the preparation of the non-reducing saccharide-forming enzyme in such a manner that they were dialyzed against a buffer containing 2M ammonium sulfate, and successively subjected to hydrophobic column chromatography and gel filtration chromatography.

DEPR:
The total enzyme activity, specific activity and yield of the non-reducing saccharide-forming enzyme in each purification step are in Table 11, while those of the **trehalose**-releasing enzyme are in Table 12.

DEPR:
A portion of a purified **trehalose**-releasing enzyme preparation, obtained by the method in Experiment 15, was electrophoresed in a 10% sodium dodecylsulfate polyacrylamide gel, and determined for molecular weight by comparing it with marker proteins commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan, revealing that it has a molecular weight of about 58,000-68,000 daltons.

DEPR:
.alpha.-Glycosyltrehalose as a substrate was prepared in accordance with the method in Experiment 4: To a 20% aqueous solution of a reducing partial starch hydrolysate selected from maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was added 2 units/g substrate, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 15, subjected to an enzymatic reaction at 40.degree. C. and pH 7.0 for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, filtered, decolored, desalted and concentrated into a high saccharide content solution which was then column chromatographed by using XT-1016 in Na.sup.+ -form, an ion-exchanger commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan. In the column chromatography, the ion-exchanger was packed in 3-jacketed stainless steel columns, having an inner diameter of 2.0 cm and a length of one m each, which were then cascaded in series, heated to give the inner column temperature of 55.degree. C., applied with 5 v/v % of the concentrated saccharide solution to the resin while keeping at 55.degree. C., and fed with 55.degree. C. hot water at SV 0.13 to obtain high-purity non-reducing saccharides having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more. Among the resultant high-purity preparations, the purities of the preparations of glucosyltrehalose, maltotriosyl-**trehalose,** maltotriosyltrehalose, maltotetraosyltrehalose and maltopentaosyltrehalose were respectively 97.6%, 98.6%, 99.6%, 98.3% and 98.1%, d.s.b.

DEPR:
An aqueous solution containing 20%, d.s.b., of one of the above 5 non-reducing saccharide preparations, namely .alpha.-glycosyltrehalose preparations, was prepared, followed by mixing it with 2 units/g substrate, d.s.b., of the purified **trehalose**-releasing enzyme obtained in Experiment 15, and subjecting the resultant to an enzymatic reaction at 40.degree. C. and pH 7.0 for 48 hours. The resultant each reaction mixture was desalted, and analyzed for saccharide composition on HPLC using "WAKOBEADS WB-T-330 column", a column of Wako Pure Chemical Industries Ltd., Tokyo, Japan. As a control, a fresh preparation of the same **trehalose**-releasing enzyme was allowed to act on maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose, and the resultant each reaction mixture was analyzed for saccharide composition on HPLC. The results are in Table 13.

DEPR:
These results confirm that the **trehalose**-releasing enzyme usable in the present invention is an enzyme which has a new reaction mechanism of specifically hydrolyzing the linkage between a **trehalose** moiety and other glycosyl moiety in .alpha.-glycosyltrehalose to release **trehalose**.

DEPR:
To purify **trehalose** in each reaction mixture, it was subjected to column chromatography using a column packed with "XT-1016", a strong-acid cation exchange resin in Na.sup.+ -form commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, followed by recovering fractions containing 97% or more of **trehalose**. The fractions were pooled and concentrated into an about 65% solution which was then allowed to stand at 25.degree. C. for 2 days to crystallize **trehalose** into hydrous crystalline **trehalose**, followed by separating and drying it in vacuo to obtain a high-purity **trehalose** preparation with a purity of 99% or more, d.s.b. The yields of **trehalose** from glucosyltrehalose, maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose and maltopentaosyltrehalose used as a substrate were respectively 9.5%, 14.9%, 16.0%, 18.5% and 17.7%, d.s.b. The high-purity **trehalose** preparations and a commercially available **trehalose** specimen as a standard were studied for melting point, heat of fusion, specific rotation, infrared absorption spectrum, powdery X-ray diffraction pattern, and readiness of hydrolysis by a trehalase specimen derived from pig kidney, commercialized by Sigma Chemical Co., St. Louise, USA. As a result, every **trehalose** preparation showed a melting point of 97.0.degree..+-.0.5.degree. C., a heat of fusion of 57.8.+-.1.2 kJ/mole and a specific rotation of +182.degree.1.1.degree., and these values well corresponded with those of the standard **trehalose** specimen, and the infrared absorption spectra and powdery X-ray diffraction patterns of the **trehalose** preparations also well corresponded with those of the standard **trehalose** specimen. Similarly as the standard **trehalose** specimen, the **trehalose** preparations were decomposed into glucose molecules. As is evident from these results, it was identified that the saccharide, which is formed by allowing the **trehalose**-releasing enzyme to act on .alpha.-glycosyltrehalose, is **trehalose**.

DEPR:
Either of the resultant reducing partial starch hydrolysates or maltotriose having a glucose polymerization degree of 3, as a substrate, was dissolved in 10 mM phosphate buffer (pH 7.0) into a one % solution which was then mixed with a purified non-reducing saccharide-forming enzyme and a purified **trehalose**-releasing enzyme, which were prepared by the method in Experiment 15, in respective amounts of 4 units/g substrate, d.s.b., and subjected to an enzymatic reaction at 40.degree. C. for 24 hours. After completion of the reaction, a portion of the resultant reaction mixture was desalted and analyzed on HPLC.

DEPR:
As is shown in Table 14, in the case of using as a substrate maltotriose having a glucose polymerization degree of 3, the **trehalose** yield after the enzymatic reactions of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme was as low as 4.2%, while in the case of using as a substrate partial starch hydrolysates having a glucose polymerization degree of 10-34.1, the **trehalose** yield was high, i.e. 66.1-80.8%. It was found that the

higher the glucose polymerization degree of the reducing partial starch hydrolysates as a substrate, the higher the purity of **trehalose** in the resultant reaction mixtures. It was also found that the purity of **trehalose** in the resultant reaction mixture can be more increased by allowing glucoamylase to act on the reaction mixtures, which were prepared by these enzymes, to hydrolyze the remaining non-reducing saccharides, having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more, into **trehalose** and glucose molecules.

DEPR:
A solution, containing one % of glycine, 10% of a high-purity **trehalose** preparation with a purity of 99.5%, d.s.b., obtained by the method in Experiment 17, and 50 mM phosphate buffer (pH 7.0), was kept at 100.degree. C. for 90 min, followed by cooling the resultant solution and determining the absorbance at a wave length of 480 nm in a 1-cm cell. As a control, glucose and maltose were similarly treated as above, and the resultants were determined for absorbance at a wave length of 480 nm. The results are in Table 15.

DEPR:
As is evident from the results in Table 15, it was revealed that the **trehalose** preparation was slightly colored on the maillard reaction, i.e. the coloration degree was only about 0.4-0.6% of that of glucose or maltose. The results show that the present **trehalose** preparation is substantially free from the maillard reaction. Thus, the preparation is a saccharide which does not substantially deteriorate amino acids even when mixed with them.

DEPR:
In accordance with the method as reported by H. Atsuji et al. in "Rinsho-Eiyo", Vol.41, No.2, pp.200-208 (1972), 30 g of a high-purity **trehalose** preparation with a purity of 99.5%, d.s.b., obtained by the method in Experiment 17 was dissolved in water into a 20 w/v % aqueous solution which was then orally administered to 6 healthy male volunteers, 26-, 27-, 28-, 29-, 30- and 31-year-old. The volunteers were collected their blood at a prescribed time interval, and each collected blood was assayed for blood sugar-and insulin-levels. As a control glucose was used. As a result, the **trehalose** preparation showed the same dynamics as glucose, i.e. the blood sugar- and insulin-levels showed their maxima at an about 0.5-1 hour after their administrations. It was revealed that the **trehalose** preparation is readily assimilated, absorbed, metabolized and utilized by the body as an energy source.

DEPR:
Similarly as in Experiment 14, a seed culture of Arthrobacter sp. Q36 (FERM BP-4316) was cultured by a fermenter for about 72 hours in place of Rhizobium sp. M-11 (FERM BP-4130). The activities of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme in the resultant culture were respectively about 1.3 units/ml and about 1.8 units/ml. Similarly as in Experiment 14, a cell suspension and a culture supernatant, prepared from the resultant culture, were assayed revealing that the former had about 0.5 units/ml of the non-reducing saccharide-forming enzyme and about 0.5 units/ml of the **trehalose**-releasing enzyme, and that the latter had about 0.8 units/ml of the non-reducing saccharide-forming enzyme and about 1.3 units/ml of the **trehalose**-releasing enzyme.

DEPR:
By using an about 18 L of a culture containing enzymes obtained by the method in Experiment 22, purified enzyme preparations were obtained similarly as the method in Experiment 15. The results in each purification step for a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme are respectively in Tables 16 and 17.

DEPR:
Purified enzyme preparations of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme, obtained as the eluates from gel filtration columns in Tables 16 and 17, were determined for purity on electrophoresis similarly as in Experiment 15. As a result, they were respectively found as a single band revealing that they were electrophoretically homogenous and relatively-high in purity.

DEPR:
A purified **trehalose**-releasing enzyme preparation, obtained by the method in Experiment 23, was determined for molecular weight on SDS-PAGE to give about 57,000-67,000 daltons. The pI of the enzyme preparation was determined on isoelectrophoresis similarly as in Experiment 3 revealing that it is about 3.6-4.6. The influences of temperature and pH on the enzyme activity, as well as the thermal stability and pH stability, were studied similarly as in Experiment 16. The results of them are respectively in FIGS. 14-17.

DEPR:
Among known microorganisms, those of the species Brevibacterium helvolum (ATCC 11822) and Micrococcus roseus (ATCC 186), which had been confirmed by the present inventors to produce the **trehalose**-releasing enzymes usable in the present invention, were respectively cultured by a fermenter at 27.degree. C. for 72 hours similarly as in Experiment 14. About 18 L of each resultant culture was similarly as in Experiment 15 subjected to a cell disrupter and centrifuged to obtain a supernatant which was then successively salted out with ammonium sulfate, dialyzed and subjected to an ion-exchange column to obtain a partially purified enzyme preparation, followed by studying the properties. The results are in Table 18 including those of Rhizobium sp. M-11 and Arthrobacter sp. Q36.

DEPR:
In accordance with the method in Experiment 25, the experiment to prepare **trehalose** by the partially purified enzyme preparations from non-reducing saccharides, having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more, was conducted. As a result, it was revealed that similarly as the **trehalose**-releasing enzyme from Rhizobium sp. M-11, all the preparations release **trehalose** from .alpha.-glycosyltrehalose.

DEPR:
To prepare high **trehalose** content saccharide compositions from starch, the influence of the combination of enzymes and the liquefaction degrees of starch were studied. A 20% corn starch suspension was mixed with 0.1% calcium carbonate, d.s.b., and the mixture was adjusted to pH 6.5, mixed with 0.1-2.0% per g starch, d.s.b., of "TERMAMYL", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, enzymatically reacted at 95.degree. C. for 15 min, and autoclaved at 120.degree. C. for 10 min into a liquefied solution (DE 2.5-20.5). The resultant mixture was promptly cooled, and then mixed with 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme prepared by the method in Experiment 2, 10 units/g starch, d.s.b., of a purified **trehalose**-releasing enzyme prepared by the method in Experiment 15, 500 units/g starch, d.s.b., of a starch debranching isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and 5 units/g starch, d.s.b., of a cyclomaltodextrin glucanotransferase specimen commercialized by Hayashibara Biochemical Laboratories, inc., Okayama, Japan, followed by the enzymatic reaction at pH 6.0 and 45.degree. C. for 24 hours. The reaction mixture was heated at 95.degree. C. for 10 min cooled, mixed with 10 units/g starch, d.s.b., of glucoamylase, and enzymatically reacted at pH 5.0 for 10 hours. The reaction mixture thus obtained was analyzed on HPLC and examined for **trehalose** content (w/w %, d.s.b.) with respect to the total carbohydrates. As a control, a liquefied starch solution was first subjected to the action of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme, then the reaction mixture was similarly as above subjected to the action of glucoamylase, followed by examining the resultant mixture on HPLC. The results are in Table 19.

DEPR:
As is evident from the results in Table 19, it was revealed that in the case of preparing high **trehalose** content saccharide compositions from starch, a relatively-low liquefaction degree of starch, preferably, a DE less than 15, more preferably, a DE less than 10, is satisfactory. It was also found that, when the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme are used in combination with starch debranching enzyme and/or cyclomaltodextrin glucanotransferase, the **trehalose** yield from starch increases up to about 2-4 fold higher than that yielded by only using the former two enzymes. Therefore, the combination use is advantageous for an

industrial-scale production of **trehalose** from starch.

DEPR:
Potato starch was prepared into an about 20 w/v % suspension which was then mixed with 0.3 w/v % oxalic acid and autoclaved, cooled and neutralized with calcium carbonate to obtain a liquefied solution with a pH of 6.5 and a DE of about 12. To the solution was added 2 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme and 300 units/g starch, d.s.b., of isoamylase, and enzymatically reacted at 45.degree. C. for 24 hours. The reaction mixture was heated to 95.degree. C. to inactivate the remaining enzyme, cooled and filtered to obtain a supernatant which was then in usual manner decolored with an activated charcoal, desalted and purified with ion-exchangers in H--and OH-form, and concentrated to obtain an about 50% syrup in a yield of about 90%, d.s.b. The product, a saccharide composition with a reduced reducibility which had a DE of about 8 and contained .alpha.-glycosyltrehalose and reducing amylaceous saccharides, was placed in an autoclave, mixed with 10% Raney nickel, and heated up to a temperature of 90.degree.-120.degree. C. while stirring, followed by increasing the hydrogen pressure to 20-120 kg/cm.sup.2 to terminate the hydrogenation. Thereafter, the Raney nickel was removed, and, in usual manner, decolored, desalted, purified and concentrated to obtain a 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Tapioca starch was prepared into an about 25% suspension which was then mixed with 0.2% per g starch, d.s.b., of "NEO-SPITASE", .alpha.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan. The resultant suspension was enzymatically reacted at 85.degree.-90.degree. C. for about 20 min, then autoclaved at 120.degree. C. and promptly cooled to obtain a liquefied solution with a DE of about 4. To the solution were added 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 9, 100 units/g starch, d.s.b., of pullulanase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and 20 units/g starch, d.s.b., of maltotetraose-forming enzyme produced by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and the mixture was enzymatically reacted at pH 6.5 and 40.degree. C. for 36 hours. The reaction mixture was similarly as in Example A-1 heated to inactivate the remaining enzyme, purified and concentrated into an about 60% solution. To increase the content of non-reducing saccharides in the solution, it was column chromatographed with "XT-1016", a strong-acid cation exchange resin in Ca.sup.2+ -form commercialized by Tokyo Organic Chemical Industries Ltd., Tokyo, Japan. The procedure was as follows: The resin was packed in 4 jacketed-stainless steel columns, having an inner diameter of 5.4 cm, which were then cascaded in series to give a total gel-bed depth of 20 m. The columns were heated to give the inner column temperature of 55.degree. C., and fed with 5 v/v % of the solution as a feed solution while keeping at the temperature, followed by fractionating it by feeding to the columns with 55.degree. C. hot water at SV 0.2 to collect fractions rich in non-reducing saccharides having a glucose polymerization degree of 4-6. The fractions thus obtained were pooled, purified and concentrated into an about 50% syrup, d.s.b. The syrup, a saccharide composition with a reduced reducibility and a DE 5.4 which contains .alpha.-glycosyltrehalose and reducing saccharides, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 50%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Corn starch was prepared into a 30% suspension which was then mixed with calcium carbonate to give the final concentration of 0.1%, d.s.b., and the resultant mixture was adjusted to pH 6.5, admixed with 0.3% per g starch, d.s.b., of "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S Copenhagen, Denmark, and subjected to an enzymatic reaction at 95.degree. C. for 15 min. The reaction mixture was autoclaved at 120.degree. C., promptly cooled into a liquefied solution (DE 4) which was then admixed with 4 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 2, 300 units/g starch, d.s.b., of isoamylase, and 5 units/g starch, d.s.b., of cyclomaltodextrin glucano-transferease commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and subjected to an enzymatic reaction at pH 6.3 and 45.degree. C. for 48 hours. The reaction mixture was heated at 95.degree. C. for 10 min, cooled, admixed with 10 units/g starch, d.s.b., of .beta.-amylase, and enzymatically reacted at 55.degree. C. and pH 5.5 for 16 hours. The reaction mixture was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into an about 50% syrup. The syrup, a saccharide composition with a reduced reducibility which contained reducing saccharides and non-reducing saccharides such as those having a **trehalose** structure as an end unit and .alpha.-glycosyl .alpha.-glucosides, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
A syrup obtained by the method in Example A-3 was prepared into an about 55% solution which was then column chromatographed with a strong-acid cation exchange resin in alkaline form in accordance with the method in Example A-2, followed by collecting fractions rich in non-reducing saccharides with a glucose polymerization degree of 3-6. The fractions were pooled, purified and concentrated into an about 50% syrup. The syrup, a saccharide composition (DE 8) with a reduced reducibility which contained reducing amylaceous saccharides and non-reducing saccharides such as .alpha.-glycosyl .alpha.-glucosides and those having a **trehalose** structure as an end unit, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 30%, d.s.b. The syrup thus obtained is a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, and has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability. Thus, it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Corn starch was prepared into an about 30% suspension, and, in accordance with the method in Example A-3, .alpha.-amylase was allowed to act on the suspension to obtain a liquefied solution (DE 4) which was then admixed with 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 2, 10 units/g starch, d.s.b., of a purified **trehalose**-releasing enzyme obtained by the method in Experiment 15, and 500 units/g starch, d.s.b., of isoamylase, and subjected to an enzymatic reaction at pH 6.0 and 40.degree. C. for 48 hours. The reaction mixture containing 76.3% **trehalose**, d.s.b., was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into an about 45% syrup. The syrup thus obtained was a non-reducing saccharide composition rich in **trehalose**, and, in accordance with the method in Example A-1, it was hydrogenated, purified and concentrated into an about 85% solution. The resultant solution was placed in a crystallizer, crystallized while gently stirring and gradually cooling, transferred to a plastic plain vessel, allowed

to stand at ambient temperature for 2 days, and aged to terminate the crystallization and to form a block. The resultant block was pulverized by a cutter to obtain a powdery saccharide composition with a reduced reducibility containing hydrous **trehalose** and sugar alcohols in a yield of 80% with respect to the material starch, d.s.b. The product with a reduced reducibility (DE of less than 1) is readily handleable and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Tapioca starch was prepared into an about 30% suspension, and, in accordance with the method in Example A-2, .alpha.-amylase was allowed to act on the suspension to form a liquefied solution (DE 5), followed by adding to the solution 3 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 10, 5 units/g starch, d.s.b., of a purified **trehalose**-releasing enzyme obtained by the method in Experiment 23, and 200 units/g starch, d.s.b., of cyclomaltodextrin glucanotransferase, and subjecting the resultant mixture to an enzymatic reaction at 45.degree. C. for 48 hours. The reaction mixture containing 84.7% **trehalose** was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and continuously crystallized while concentrating. The resultant massecuite was separated by a basket-type centrifuge, and the resultant crystal was washed by spraying thereto a small amount of water to obtain a crystalline **trehalose** hydrate in a yield of about 55%, d.s.b. The resultant mother liquor, containing relatively-large amounts of reducing amylaceous saccharides, **trehalose** and non-reducing saccharide having a **trehalose** structure, was concentrated into a 50% syrup of saccharides with a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 30%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains **trehalose,** sugar alcohols and non-reducing saccharides having a **trehalose** structure, has a mild and high-quality sweetness, relatively-low viscosity, and satisfactory moisture-retaining ability. Thus, it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stability, filler, excipient and/or diluent in a variety of compositions.

DEPR:
The heat inactivated reaction mixture in Example A-6 was mixed with 10 units/g substrate, d.s.b., of glucoamylase, and subjected to an enzymatic reaction at pH 5.0 and 50.degree. C. for 10 hours. The resultant reaction mixture was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into a 45% syrup with a high **trehalose** content and a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated and purified into an about 70% solution which was then placed in a crystallizer and crystallized while stirring and gradually cooling to obtain a massecuite with a crystallization percentage of about 40%. The massecuite was sprayed at a pressure of 150 kg/cm.sup.2 from a nozzle mounted on the top of a drying tower while 85.degree. C. hot air was blowing to the contents from the upper part of the drying tower and collecting the resultant crystalline powders on a wire netting conveyer provided in the basement of the drying tower. The crystalline powders were gradually transferred out of the drying tower and recovered while 45.degree. C. hot air was blowing to the powders through under the conveyer. The crystalline powders thus obtained were placed in an aging tower and aged for 10 hours while a hot air was blowing to the contents to terminate the crystallization and drying. Thus, a saccharide powder with a reduced reducibility, which contained hydrous **trehalose** crystal and sorbitol, was obtained in a yield of about 75% with respect to the material starch, d.s.b. The powder with a reduced reducibility (DE of less than 1) is readily handleable and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
A mutant of Rhizobium sp. M-11 (FERM BP-4130) was inoculated in a nutrient culture medium and cultured in a fermenter for about 70 hours in accordance

with the method in Experiment 1. The resultant culture was filtered with an SF membrane to remove cells to obtain an about 100 L supernatant which was then concentrated with a UF membrane into an about 5 L enzyme concentrate containing about 410 units/ml of a non-reducing saccharide-forming enzyme and about 490 units/ml of a **trehalose**-releasing enzyme. Corn starch was prepared into an about 33% suspension which was then treated with .alpha.-amylase to obtain a liquefied solution (DE of about 4) in accordance with the method in Example A-3, mixed with 0.02 ml per g starch, d.s.b., of the concentrated enzyme solution, 500 units/g starch, d.s.b., of isoamylase, and 5 units/g starch, d.s.b., of cyclomaltodextrin glucanotransferase, and enzymatically reacted at pH 6.2 and 40.degree. C. for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, mixed with 10 units/g substrate, d.s.b., of glucoamylase, and enzymatically reacted at pH 5.0 and 50.degree. C. for 10 hours. The resultant mixture containing 85.6% **trehalose,** d.s.b., was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated to obtain a 45% syrup of saccharides rich in **trehalose** with a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated, purified, and, in accordance with the method in Example A-5, concentrated and crystallized to form a block which was then pulverized with a cutter to obtain a saccharide powder with a reduced reducibility, which contains hydrous **trehalose** crystal and sorbitol, in a yield of about 80% with respect to the material starch, d.s.b. The product with a reduced reducibility (DE of less than 1) is readily handleable and can be used in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Into a fermenter was poured a liquid nutrient culture medium consisting of 2 w/v % glucose, 0.5 w/v % polypeptone, 0.1 w/v % yeast extract, 0.1 w/v % dipotassium phosphate, 0.06 w/v % sodium dihydrogen phosphate, 0.05 w/v % magnesium sulfate, 0.5 w/v % calcium carbonate, and water, and the medium was sterilized by heating, cooled and inoculated with a seed culture of Pimelobacter sp. R48 (FERM BP-4315), followed by the incubation at 27.degree. C. for about 40 hours under stirring conditions. The resultant culture had 0.55 units/ml of a maltose-**trehalose** converting enzyme. 0.18 kg of wet cells collected from 18 L of the culture was suspended in 10 mM phosphate buffer (pH 7.0), and about 1.5 L of the suspension was treated with an ultrasonic cell disrupter to disrupt cells. The resultant mixture was centrifuged to obtain a supernatant which was then concentrated with a UF membrane to obtain an about 500 ml of a concentrated enzyme solution containing about 18 units/ml of a maltose-**trehalose** converting enzyme. To 15% corn starch suspension (pH 5.5) was added 2 units/g starch, d.s.b., of "SPITASE HS", .alpha.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and the mixture was stirred, heated to effect gelatinization and liquefaction, and then promptly autoclaved at 120.degree. C. for 20 min. Thereafter, the resultant mixture was cooled to 55.degree. C., adjusted to pH 5.0, mixed with 300 units/g starch, d.s.b., of isoamylase, 20 units/g starch, d.s.b., of .beta.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and enzymatically reacted for 24 hours to obtain an about 92% maltose solution. The solution thus obtained was heated at 100.degree. C. for 20 min, cooled to 20 .degree. C., adjusted to pH 7.0, and mixed with 1.5 units/g starch, d.s.b., of the concentrated enzyme solution prepared in the above, and enzymatically reacted for 72 hours. The resultant reaction mixture was heated at 95.degree. C. for 10 min, cooled, and, in usual manner, decolored with an activated charcoal, filtered, desalted and purified with ion-exchangers in H--and OH-form, and concentrated into an about 50% syrup.

DEPR:
The product contained about 64% **trehalose,** d.s.b., and had a low DE of 18.0. In accordance with the method in Example A-1, the syrup was hydrogenated, purified and concentrated to obtain an about 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide syrup with a reduced reducibility (DE of less than 1) which contains **trehalose,** maltitol and a small amount of sorbitol, has a mild sweetness, adequate viscosity, and satisfactory moisture retaining ability, and these render it arbitrarily useful in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Thirty-three parts by weight of a powdered orange juice prepared by spray

drying was mixed to homogeneity under stirring conditions with 50 parts by weight of a powdery saccharide composition with a reduced reducibility obtained by the method in Example A-5, 10 parts by weight of sucrose, 0.65 parts by weight of anhydrous citric acid, 0.1 part by weight of malic acid, 0.1 part by weight of L-ascorbic acid, 0.1 part by weight of sodium citrate, 0.5 parts by weight of pullulan, and an adequate amount of a powdered flavor. The resultant mixture was pulverized, fed to a fluidized-bed granulator and granulated for 30 min by spraying to the contents a high **trehalose** content syrup, as a binder, obtained by the method in Example A-6 while 40.degree. C. air was sending to the contents. The granules thus obtained were weighed and packed to obtain the desired product. The product, a powdered juice containing about 30% orange juice, d.s.b., was stable for a relatively-long period of time without giving an unsatisfactory smell and taste.

DEPR:
Two hundred parts by weight of a powdery saccharide composition with a reduced reducibility, obtained by the method in Example A-5, and 300 parts by weight of maltose were mixed with 3 parts by weight of iodine dissolved in 50 parts by weight of methanol, and the resultant solution was admixed with 200 parts by weight of 10 w/v % pullulan solution to obtain the desired product with an adequate spreadability and adhesiveness. The product exerts a bactericidal activity due to the iodine and acts as an energy-supplementing agent for living cells due to the **trehalose,** and therefore, it can shorten the healing period and readily cure the wounded sites. [Effect on the Invention]

DEPR:
As is evident from above, the present saccharide composition, which comprises sugar alcohols and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, has a satisfactory stability and a high-quality sweetness. The saccharide composition is assimilated, absorbed and utilized by the body when orally administered. More particularly, the **trehalose** contained in the saccharide composition is readily metabolized and utilized by the body. Thus, the present saccharide composition can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals. The reducing saccharides with a reduced reducibility used as a material for the present saccharide composition include (i) those prepared by allowing a non-reducing saccharide-forming enzyme together with a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on a liquefied starch solution whereby non-reducing saccharides such as **trehalose** and saccharides having a **trehalose** structure are formed in an increased yield to obtain the objective saccharide composition with a reduced reducibility and a relatively-low molecular weight and viscosity, and (ii) those prepared by allowing a maltose-**trehalose** converting enzyme to act on maltose to form a saccharide composition of maltose and **trehalose**. These saccharide compositions are satisfactorily used as a material for the present invention and facilitate the industrial-scale preparation of the present invention.

DEPL:
Production of **trehalose**-releasing enzyme by Rhizobium sp. M-11

DEPL:
Property of **trehalose**-releasing enzyme

DEPL:
Preparation of **trehalose** by .alpha.-glycosyltrehalose

DEPL:
Preparation of **trehalose** from reducing partial starch hydrolysates

DEPL:
Production of **trehalose**-releasing enzyme by Arthrobacter sp. Q36

DEPL:
Preparation of **trehalose** from .alpha.-glycosyltrehalose By using a purified enzyme preparation obtained by the method in Experiment 23, **trehalose** was prepared from non-reducing saccharides having a **trehalose** structure as an end

unit and a glucose polymerization degree of 3 or more according to the method in Experiment 17 revealing that the enzyme preparation releases **trehalose** from .alpha.-glycosyltrehalose similarly as the **trehalose**-releasing enzyme derived from Rhizobium sp. M-11.

DEPL:
Production and property of **trehalose**-releasing enzyme by known microorganism

DEPL:
Influence of starch liquefaction decree and enzyme for preparing high **trehalose** content saccharide composition

DEPV:
Specifically hydrolyzing the linkage between a **trehalose** moiety and other glycosyl moiety;

DEPV:
Converting maltose into **trehalose,** and vice versa;

DEPV:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is phydrolyzed by glucoamylase into one **trehalose** molecule and 2 glucose molecules.

DEPV:
1. The **trehalose**-releasing enzyme specifically hydrolyzes the linkage between a **trehalose** moiety and a glycosyl moiety in .alpha.-glycosyltrehalose to form **trehalose** and a reducing saccharide having a glucose polymerization degree of one or more; and

DEPV:
2. Maltooligosaccharide is not hydrolyzed by the **trehalose**-releasing enzyme.

DETL:
TABLE 5 ______________________________________ Saccharide Glucose **Trehalose** Molecular ratio preparation (%) (%) (Glucose/Trehalose) ______________________________________ P I 36.2 63.8 1.07 P II 52.0 48.0 2.06 P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11 ______________________________________

DETL:
TABLE 7 ______________________________________ Saccharide composition of hydrolysate by .alpha.-glucosidase Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________________ P I 36.5 63.0 0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3 0.3 ______________________________________

DETL:
TABLE 8 ______________________________________ Saccharide composition of hydrolysate by rat intestinal acetone powder Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________________ P I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P V 73.4 26.5 0.1 ______________________________________

DETL:
TABLE 13 ______________________________________ Elution time Percen- on HPLC tage Substrate Product (min) (%) ______________________________________ Glucosyltrehalose **Trehalose** 27.4 17.5 Glucose 33.8 6.5 Glucosyltrehalose 23.3 76.0 Maltosyltrehalose **Trehalose** 27.4 44.3 Maltose 28.7 44.4 Maltosyltrehalose 21.6 11.3 Maltotriosyltrehalose **Trehalose** 27.4 39.5 Maltotriose 25.9 60.0 Maltotriosyltrehalose 19.7 0.5 Maltotetraosyltrehalose **Trehalose** 27.4 34.2 Maltotetraose 24.1 65.5 Maltotetraosyltrehalose 18.7 0.3 Maltopentaosyltrehalose **Trehalose** 27.4 29.1 Maltopentaose 22.6 70.6 Maltopentaosyltrehalose 17.8 0.3 Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100 ______________________________________

DETL:
TABLE 14 ______________________________________ Glucose polymerization degree of reducing partial starch hydro- Composition (%) lysate Reaction product A B ________________________________ 34.1 **Trehalose** 80.8 83.5 Glucose 0.2 16.5 Reducing oligosaccharides 14.4 0.0 Glucosyltrehalose 4.6 0.0 26.2 **Trehalose** 79.7 82.5 Glucose 0.2 17.5 Reducing oligosaccharides 15.3 0.0 Glucosyltrehalose 4.8 0.0 18.1 **Trehalose** 77.7 80.7 Glucose 0.2 19.3 Reducing oligosaccharides 17.0 0.0 Glucosyltrehalose 5.1 0.0 15.2 **Trehalose** 75.0 78.5 Glucose 0.3 21.5 Reducing oligosaccharides 18.6 0.0 Glucosyltrehalose 6.1 0.0 10.0 **Trehalose** 66.1 70.1 Glucose 0.3 29.9 Reducing oligosaccharides 27.6 0.0 Glucosyltrehalose 7.7 0.0 3 **Trehalose** 4.2 20.8 (Maltotriose) Glucose 2.1 79.2 Maltotriose 65.0 0.0 Glucosyltrehalose 28.7 0.0 ______________________________________ Note : In the Table, the symbol "A" means a composition after enzymatic reaction of a nonreducing saccharideforming enzyme and a trehalosereleasing enzyme, and the symbol "B" means a composition after enzymatic reaction of glucoamylase. The wording "Glucosyltrehalose" means a nonreducing saccharide having a **trehalose** structure as an end unit and glucose polymerization degree of 3 or more.

DETL:
TABLE 15 ______________________________________ Coloration degree Saccharide preparation (480 nm) ______________________________________ **Trehalose** (Present invention) 0.006 Glucose (Control) 1.671 Maltose (Control) 0.926 ______________________________________

CLPR:
2. The process as claimed in claim 1, wherein said saccharide mixture is obtainable by allowing a non-reducing saccharide-forming enzyme together with or without a **trehalose**-releasing enzyme to act on a reducing partial starch hydrolysate.

CLPR:
4. The process as claimed in claim 1, wherein said saccharide mixture is obtainable by allowing a maltose-**trehalose** converting enzyme to act on a reducing partial starch hydrolysate containing maltose.

CLPR:
9. A method for reducing the reducibility of a saccharide mixture with a reduced reducibility which comprises a reducing amylaceous saccharide and a non-reducing saccharide selected from the group consisting of **trehalose,** a saccharide having a **trehalose** structure within the molecule, a saccharide having a **trehalose** structure as an end unit, and mixtures thereof, which contains a step of hydrogenating said saccharide mixture to form a saccharide composition comprising said non-reducing saccharide and a formed sugar alcohol.

CLPV:
hydrogenating a saccharide mixture comprising a reducing amylaceous saccharide and a non-reducing saccharide selected from the group consisting of **trehalose,** a saccharide having a **trehalose** structure within the molecule, a saccharide having a **trehalose** structure as an end unit, and mixtures thereof; and

CLPV:
either allowing a non-reducing saccharide-forming enzyme together with a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase, or allowing a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme together with a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on a liquified starch solution;

CLPV:
hydrogenating the resultant saccharide mixture with a reduced reducibility containing a reducing amylaceous saccharide and a non-reducing saccharide selected from the group consisting of **trehalose,** a saccharide having a **trehalose** structure within the molecule, a saccharide having a **trehalose** structure as an end unit, and mixtures thereof; and

ORPL:
Hoelzle, Inger and John G. Streeter; "Increased Accumulation of **Trehalose** in

Rhizobia Cultured Under 1% Oxygen"; Applied Environmental Biology; vol. 56, No. 10; pp. 3213-3215; Oct. 1990.

USPT

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DOCUMENT-IDENTIFIER: US 5723327 A
TITLE: Thermostable **trehalose**-releasing enzyme, and its preparation and uses
DATE-ISSUED: March 3, 1998
INVENTOR-INFORMATION:

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Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
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ABSTRACT:
Disclosed are novel thermostable **trehalose**-releasing enzyme, and its preparations and uses. The enzyme is obtainable from the culture of microorganisms such as **Sulfolobus acidocaldarius** (ATCC 33909 and ATCC 49426) and **Sulfolobus** solfataricus (ATCC 35091 and ATCC 35092), and capable of hydrolyzing at a temperature of over 55.degree. C. the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher. **Trehalose** and compositions containing the same are extensively useful in food products, cosmetics and pharmaceuticals.
15 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

TTL:
Thermostable **trehalose**-releasing enzyme, and its preparation and uses

ABPL:
Disclosed are novel thermostable **trehalose**-releasing enzyme, and its preparations and uses. The enzyme is obtainable from the culture of microorganisms such as **Sulfolobus acidocaldarius** (ATCC 33909 and ATCC 49426) and **Sulfolobus** solfataricus (ATCC 35091 and ATCC 35092), and capable of hydrolyzing at a temperature of over 55.degree. C. the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher. **Trehalose** and compositions containing the same are extensively useful in food products, cosmetics and pharmaceuticals.

BSPR:
The present invention relates to a thermostable **trehalose**-releasing enzyme, and its preparation and uses, more particularly, to a novel thermostable **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and the remaining glycosyl moiety in non-reducing saccharides having a **trehalose** structure as an end unit and having a glucose polymerization degree of 3 or higher, and to the preparation of the enzyme. The present invention further relates to **trehalose** obtainable by using the enzyme and to compositions containing the same.

BSPR:
Trehalose or .alpha., .alpha.-**trehalose** is known as a non-reducing saccharide consisting of glucose units. As is described in Advances in Carbohydrate Chemistry, Vol. 18, pp. 201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol. 56, pp. 3,213-3,215 (1990), **trehalose** widely exists in microorganisms, mushrooms, insects, etc., though the content is relatively low. **Trehalose** is a non-reducing saccharide, so that it neither reacts with substances containing amino groups such as amino acids and proteins, induces the amino-carbonyl reaction, nor deteriorates amino acid-containing substances. Thus, **trehalose** is expected to be used without fear of causing an unsatisfactory browning and deterioration. Because of these, the establishment of the industrial-scale preparation of **trehalose** has

been in great demand.

BSPR:
Conventional preparations of **trehalose** are, for example, those which are disclosed in Japanese Patent Laid-Open No. 154,485/75 wherein microorganisms are utilized, and reported in Japanese Patent Laid-Open No. 216,695/83 wherein maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** contained in microorganisms used as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, unless otherwise specified), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, the concentration of maltose as a substrate could not be set to a desired level; (ii) the enzymatic reaction systems of the phosphorylases are reversible reactions, and their yields of the objective **trehalose** are relatively low; and (iii) it is substantially difficult to retain their reaction systems stably and to continue their enzymatic reactions smoothly. Thus, these conventional preparations have not been actually used as an industrial-scale preparation.

BSPR:
Considering the aforementioned circumstances, the present inventors have energetically studied enzymes which are capable of forming saccharides having a **trehalose** structure when allowed to act on starch hydrolysates. As a result, the present inventors found that Rhizobium sp. M-11 or Arthrobactor sp. Q36 is capable of producing a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, and simultaneously found that a **trehalose**-releasing enzyme produced by Rhizobium sp. M-11 or Arthrobactor sp. Q36 can hydrolyse the non-reducing saccharides into **trehalose** and glucose and/or maltooligosaccharide at a constant amount. These enzymes realized that an objective amount of **trehalose** can be readily obtained by using starch as a material, and the aforementioned object concerning a **trehalose** is expected to be attainable.

BSPR:
Enzymes derived from Rhizobium sp. M-11 or Arthrobactor sp. Q36, however, are relatively-low in thermal stability. Thus, in case that these enzymes are utilized for preparing **trehalose** and non-reducing saccharides having a **trehalose** structure as an end unit, it is necessary to allow the enzymes to act on at a temperature of below 55.degree. C. With regard to the temperature of enzymatic reaction, as described in the column titled "Enzymes related to saccharides" in the chapter titled "Enzymes related to saccharides and their applications" in "Koso-Ouyou-no-Chishiki" (Knowledge on Enzyme Applications), the first edition, pp. 80-129 (1986) that "In the conditions of industrial-scale enzymatic reactions for saccharification, the reactions at a temperature of below 55.degree. C. involves a risk of contamination and a decrease of pH during the reaction, in long-time enzymatic reactions using starch as a material, when an enzyme is allowed to act on at a temperature of below 55.degree. C., because of contamination and a decrease of pH of reaction mixtures which inactivate the activity of such enzymes, and it is necessary to add lysozyme for the prevention of contamination and the pH control of the reaction mixtures. In addition, when the hydrolysis of partial starch hydrolysates is relatively low, insoluble substances may be formed due to retrogradation of starch.

BSPR:
On the other hand, since a thermostable enzyme can maintain its activity at a relatively-high temperature, contamination during the enzymatic reaction is less of a concern and the retrogradation of partial starch hydrolysates is scarcely caused. As a source of thermostable enzymes, thermophilic microorganisms can be generally considered. Regarding a preparation of **trehalose** using thermophilic microorganisms, as described in Biotechnology Letters, Vol. 12, pp. 431-432 (1990) and Biotech Forum Europe, Vol. 8, pp. 201-203 (1991), it was reported that the partially purified enzyme preparation obtainable from the cell and cell extract of **Sulfolobus** solfataricus (ATCC

49155) forms glucose and **trehalose** when allowed to act on substrate such as amylose and soluble starch. A purification of such an enzyme preparation can not be completed, however, as the physicochemical properties of the enzyme thus prepared are not sufficiently indicated and the action of the enzyme has not been clarified, and only a preparation of **trehalose** is indicated. Thus, there has been a great demand to establish a novel preparation of **trehalose** by utilizing a thermostable enzyme capable of acting at a temperature of over 55.degree. C.

BSPR:
The present invention is to provide a novel preparation of **trehalose** to form **trehalose** or a saccharide composition containing the same. The **trehalose** is can be prepared from reducing partial starch hydrolysates by a thermostable **trehalose**-releasing enzyme which is capable of acting at a temperature of over 55.degree. C. and clarifying its action, and to **trehalose** obtainable by said preparation and a saccharide composition containing the same as well as their uses.

BSPR:
In order to attain the aforementioned object, the present inventors, desiring an establishment of a novel thermostable enzyme which can release **trehalose** from non-reducing saccharides having a **trehalose** structure and having a degree of glucose polymerization of 3 or higher, have extensively screened microorganisms capable of producing said enzyme while centering around thermophilic microorganisms.

BSPR:
As a result, the present inventors found that microorganisms of the genus **Sulfolobus**, named as "**Sulfolobus acidocaldarius**" ATCC 33909 and ATCC 49426, and as "**Sulfolobus** solfataricus" ATCC 35091 and ATCC 35092, these as disclosed in Japanese Patent Application No. 166,011/94, produce a thermostable non-reducing saccharide-forming enzyme and also a novel thermostable **trehalose**-releasing enzyme which are capable of acting at a temperature of over 55.degree. C., and found that the objective preparation of **trehalose** at a temperature of over 55.degree. C. is readily conducted by allowing the thermostable non-reducing saccharide enzyme together with this novel thermostable **trehalose**-releasing enzyme to act on reducing partial starch hydrolysates. The present inventors also found that **trehalose** is readily preparable by allowing the thermostable non-reducing saccharide-forming enzyme together with the novel thermostable **trehalose**-releasing enzyme to act on reducing partial starch hydrolysates and subjecting to the action of glucoamylase or .alpha.-glucosidase to obtain reacted solutions containing **trehalose** with a relatively-high purity. Thus, the present inventors accomplished this invention.

DRPR:
FIG. 1 shows elution patterns of the present thermostable **trehalose**-releasing enzyme and a non-reducing saccharide-forming enzyme eluted from a column packed with a gel of "DEAE-TOYOPEARL.RTM.".

DRPR:
FIG. 2 shows the influence of temperature on the activity of the present thermostable **trehalose**-releasing enzyme.

DRPR:
FIG. 3 shows the influence of pH on the activity of the present thermostable **trehalose**-releasing enzyme.

DRPR:
FIG. 4 shows the influence of temperature on the stability of the present thermostable **trehalose**-releasing enzyme.

DRPR:
FIG. 5 shows the influence of pH on the stability of the present thermostable **trehalose**-releasing enzyme.

DEPR:
The present invention relates to a novel thermostable **trehalose**-releasing enzyme, and its preparation and uses. The present invention further relates to

a microorganism capable of producing said enzyme, **trehalose** prepared with said enzyme, and compositions containing the same.

DEPR:
The present inventors have extensively screened microorganisms capable of producing a novel thermostable **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and the remaining glycosyl moiety in non-reducing saccharides having a **trehalose** structure and having a glucose polymerization degree of 3 or higher, and eventually found the objective microorganisms.

DEPR:
Now, the present inventors found that microorganisms of the genus **Sulfolobus,** named as "**Sulfolobus acidocaldarius**" ATCC 33909 and ATCC 49426, and as "**Sulfolobus** solfataricus" ATCC 35091 and ATCC 35092, are capable of producing a novel thermostable **trehalose**-releasing enzyme.

DEPR:
In addition to the above-mentioned microorganisms, other strains of the genus **Sulfolobus** and their mutants can be arbitrarily used in the present invention as long as they produce a thermostable **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher.

DEPR:
Any nutrient culture medium can be used in the invention as long as these microorganisms can grow therein and produce the present thermostable **trehalose**-releasing enzyme: For example, synthetic- and natural-nutrient culture media can be used as the nutrient culture medium. Any carbon-containing substance can be used in the present invention as a carbon source as long as it is utilized by the microorganisms: Examples of such a carbon source are saccharides such as glucose, fructose, lactose, sucrose, mannitol, sorbitol, molasses and reducing partial starch hydrolysates; and organic acids such as citric acid, succinic acid and their salts. The concentrations of these carbon sources in nutrient culture media are appropriately chosen. For example, in the case of using reducing partial starch hydrolysates, a preferable concentration is usually 20% or lower, more particularly, 5% or lower, d.s.b., in view of the growth of microorganisms. The nitrogen sources usable in the present invention are, for example, inorganic nitrogen compounds such as ammonium salts and nitrates; and organic nitrogen-containing substances such as urea, corn steep liquor, casein, peptone, yeast extract and beef extract. The inorganic ingredients usable in the invention are, for example, calcium salts, magnesium salts, potassium salts, sodium salts, phosphates and other salts of manganese, zinc, iron, copper, molybdenum and cobalt.

DEPR:
The present thermostable **trehalose**-releasing enzyme thus obtained has the following physicochemical properties:

DEPR:
The activity of the present thermostable **trehalose**-releasing enzyme is assayed as follows: One ml of an enzyme solution is added to 4 ml of 1.25 w/v % maltotriosyltrehalose (.alpha.-maltotetraosyl .alpha.-glucoside) in 50 mM phosphate buffer (pH 6.0) as a substrate, and the mixture solution is incubated at 60.degree. C. for 30 min. The reaction solution is mixed with Somogyi copper liquor to suspend the enzymatic reaction, and followed by determining the reducing power of the solution on the Somogyi-Nelson's method. As a control, an enzyme solution, which had been heated at 100.degree. C. for 30 min to inactivate any enzyme activity, is treated similarly as above. With such a determination, one unit activity of the present enzyme is defined as the amount of enzyme which increases the reducing power of that of one micromole of glucose per minute.

DEPR:
Non-reducing saccharides which can be used as a substrate for the present

enzyme are those having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher. Examples of such a substrate are glucosyltrehalose, maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose and maltopentaosyltrehalose which are obtainable by allowing a non-reducing saccharide-forming enzyme to act on maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. In addition, relatively-low reducing partial starch hydrolysates containing non-reducing saccharides which have a **trehalose** structure and a degree of glucose polymerization of 3 or higher, those prepared by allowing non-reducing saccharide-forming enzyme to act on reducing partial starch hydrolysates which are preparable by partially hydrolyzing amylaceous substances such as starch, amylopectin and amylose by amylases or acids, can be used.

DEPR:
As non-reducing saccharide-forming enzymes which forms non-reducing saccharides having a **trehalose** structure and having a degree of glucose polymerization of 3 or higher when allowed to act on reducing partial starch hydrolysates, those derived from Rhizobium sp. M-11 or Arthrobactor sp. Q36 as disclosed in Japanese Patent Application No. 349,216/93, can be used, however, in case that an enzymatic reaction proceeds at a temperature of over 55.degree. C., the thermostable non-reducing saccharide-forming enzyme which belongs to the group of the genus **Sulfolobus,** disclosed in Japanese Patent Application No. 166,011/94, can be used favorably.

DEPR:
The concentration of substrates in the present invention is not specifically restricted. For example, in the case of using 0.1% or 50% solution of a substrate, the present enzymatic reaction proceeds to form a **trehalose**. Further a solution containing the excess amount of the substrate which is not dissolved completely can be used in the present invention. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 85.degree. C., preferably, a temperature in the range of 55.degree.-70.degree. C. The reaction pH used in the present enzymatic reaction is controlled to in the range of 4-10, preferably, in the range of about 5-7. The reaction time used in the present enzymatic reaction is adequately chosen depending on the conditions of the enzymatic reaction.

DEPR:
A method of preparing a **trehalose** using reducing partial starch hydrolysates, according to the present invention, can prepare a remarkably increased amount of **trehalose,** in comparison with those disclosed in the specification of Japanese Patent Application No. 349,216/93, more particularly, contrasting with reaction solutions obtainable by the action of a non-reducing saccharide-forming enzyme together with glucoamylase. More particularly, the preparation percentage of **trehalose** obtainable by the action of a non-reducing saccharide-forming enzyme together with glucoamylase is about 30%, while that of **trehalose** obtainable by the reaction of a non-reducing saccharide-forming enzyme together with **trehalose**-releasing enzyme in the present invention is about 60% or higher.

DEPR:
The enzymatic reaction in the present invention is as follows: At first, one molecule of reducing partial starch hydrolysate having a degree of glucose polymerization of 3 or higher is converted into one molecule of non-reducing saccharide having a **trehalose** structure as an end unit by the action of non-reducing saccharide-forming enzyme, and further the resultant non-reducing saccharides are converted by the hydrolytic action of **trehalose**-releasing enzyme into one molecule of **trehalose** and one molecule of reducing partial starch hydrolysates of which a degree of glucose polymerization is decreased by 2. In case that reducing partial starch hydrolysates thus newly produced have a degree of glucose polymerization of 3 or higher, they are converted into non-reducing saccharides having a **trehalose** structure as an end unit by the action of non-reducing saccharide-forming enzyme, and followed by subjecting them to the action of **trehalose**-releasing enzyme to form one molecule of **trehalose** and reducing partial starch hydrolysates. By repeating these actions of non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme, several molecules of **trehalose** can be prepared from one molecule of reducing

partial starch hydrolysates.

DEPR:
In the aforementioned enzymatic reaction, a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme of the present invention can be allowed to act simultaneously on reducing partial starch hydroylsates having a degree of glucose polymerization of 3 or higher, and at first a non-reducing saccharide-forming enzyme is allowed to act on said reducing partial starch hydroylsates, followed by subjecting to the action of **trehalose**-releasing enzyme of the present invention. If necessary, glycoamylase is allowed favorably to act on to increase the content of **trehalose**.

DEPR:
The resultant reaction mixtures are in the usual manner subjected to filtration and centrifugation to remove insoluble substances, and the resultant solutions are decolored with an activated charcoal, desalted with ion exchangers in H- and OH-form, and concentrated into syrupy products which can be dried into powdery products. If necessary, the powdery products can be readily prepared into non-reducing saccharides with the highest possible purity by purifying the powdery products. Examples of column chromatographic fractionations such as ion-exchange column chromatography, column chromatography using an activated charcoal or a silica gel; separations using organic acids such as alcohols and acetone; and alkaline treatments to decompose and remove the remaining reducing saccharides, and with these purifications, high-purity **trehalose** products are readily obtainable.

DEPR:
If necessary, the present non-reducing saccharides having a **trehalose** structure thus obtained can be hydrolyzed by amylases such as .alpha.-amylase, .alpha.-amylase, glucoamylase, .alpha.-glucosidase and trehalase, or subjected to a saccharide-transfer reaction by using cyclomaltodextrin glucanotransferase and/or glucosyltransferase to control their sweetness and reducing power as well as to reduce their viscosity. Furthermore, the saccharide products can be arbitrarily hydrogenated to convert them into sugar alcohols to eliminate their reducing power. From the resultant products glucose can be removed by using aforesaid purification methods such as ion-exchange column chromatography to prepare high **trehalose** content fractions. The fractions thus obtained can be arbitrarily purified and concentrated into syrupy products, and, if necessary the syrupy products can be further concentrated into supersaturated solutions and crystallized to obtain hydrous crystalline **trehalose** or anhydrous crystalline **trehalose**.

DEPR:
The ion-exchange column chromatographic techniques usable in the invention include, for example, those which use a strong-acid cation-exchange resin as disclosed in Japanese Patent Laid-Open Nos. 23,799/83 and 72,598/83. By using these techniques, concomitant saccharides contained in crude **trehalose** products can be readily removed to obtain high **trehalose** content products. In this case, any one of fixed-bed, moving bed and semi-moving methods can be arbitrarily employed.

DEPR:
To prepare hydrous crystalline **trehalose**, for example, an about 65-90% solution of **trehalose** with a purity of about 60% or higher, d.s.b., is placed in a crystallizer, if necessary, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10.degree.-90.degree. C., to obtain a massecuite containing hydrous crystalline **trehalose**. Also, the continuous crystallization to prepare hydrous crystalline **trehalose** while concentrating a solution of **trehalose** under reduced pressure can be favorably used in the present invention. Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the present invention to prepare from the massecuite hydrous crystalline **trehalose** or crystalline saccharides containing the same.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline **trehalose** from the mother liquor,

and, if necessary, the hydrous crystalline **trehalose** is washed by spraying thereto with a small amount of cold water to facilitate the preparation of hydrous crystalline **trehalose** with an increased purity. In the case of spray drying, crystalline saccharides with no or substantially no hygroscopicity are readily prepared by spraying massecuites with a concentration of 60-85%, d.s.b., and a crystallization percentage of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60.degree.-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto 30.degree.-60.degree. C. hot air. In the case of block pulverization, crystalline saccharides with no or substantially no hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallization percentage of about 10-60%, d.s.b., to stand for a period from about several hours to 3 days to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks. Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into anhydrous one, it is generally prepared by placing a high **trehalose** content solution with a moisture content less than 10% in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50.degree.-160.degree. C., preferably, a temperature in the range of 80.degree.-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** at a relatively high temperature by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present **trehalose** thus obtained is stable and substantially free of reducing power, and can be mixed and processed with other materials, specifically, amino acids and amino acid-containing substances such as oligopeptides and proteins without fear of causing unsatisfactory browning and smell as well as deterioration of the materials. **Trehalose** per se has a satisfactorily-high quality and sweetness. Since **trehalose** is readily hydrolyzed by trehalase into glucose units, it is assimilated, absorbed and utilized by living bodies as a caloric source when orally administered. Furthermore, **trehalose** is not substantially fermented by dental carries-inducing microorganisms, and this renders it useful as a sweetener substantially free of inducing dental caries.

DEPR:
Trehalose can be utilized parenterally as a liquid feeding and infusion without fear of toxicity and side effects, preferably, utilized as an energy source by the body. **Trehalose** is a stable sweetener, and, especially crystalline **trehalose** is arbitrarily used as a sugar coating agent for tablets when used in combination with a binder such as pullulan, hydroxyethyl starch or polyvinylpyrrolidone. In addition, **trehalose** has properties such as osmotic pressure-controlling ability, filler-imparting ability, gloss-imparting ability, moisture-retaining ability, viscosity-imparting ability, ability to prevent crystallization of other saccharides, substantially no fermentability, and ability to prevent retrogradation of gelatinized starch.

DEPR:
Thus, the present **trehalose** and saccharide composition containing the same can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler in a variety of compositions such as food products, cigarettes, tobaccos, feeds, cosmetics and pharmaceuticals.

DEPR:
The present **trehalose** and saccharide compositions containing the same can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, sorbitol, maltitol, lactitol, dihydrochalcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:

The present **trehalose** and saccharide compositions containing the same in the form of a powder or a crystal can be used intact, or, if necessary they can be mixed with an excipient, diluent, filler and binder and formed into granules, spheres, shot-rods, plates, cubes and tablets, prior to their use. The present **trehalose** and saccharide compositions containing the same well harmonize with other materials having sourness, acidity, saltiness, bitterness, astringency and deliciousness-tastes, and have a relatively-high acid tolerance and heat resistance. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present **trehalose** and saccharide compositions containing the same can be used in seasonings such as soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake), "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare" (a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
The present **trehalose** and saccharide compositions containing the same can be also used freely for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker), "arare-mochi" (a rice-cake cube), "okoshi" (a millet-and-rice cake), "mochi" (a rice paste), "manju" (a bun with a bean-jam), "uiro" (a sweet rice jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly, pao de Castella and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickles and pickled products such as "fukujin-zuke" (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish ham, fish sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tempura" (a Japanese deep-fat fried fish paste); "chinmi" (relish) such as "uni-no-shiokara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle), "saki-surume" (dried squid strips) and "fugu-no-mirin-boshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk products such as yoghurt and cheese; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liquors; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix and "sokuseki-shiruco" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and beverages such as baby foods, foods for therapy, and beverages supplemented with nutrition; as well as for improving the tastes and qualities of the aforementioned food-products.

DEPR:
The present **trehalose** and saccharide compositions containing the same can be also used in feeds and pet foods for animals such as domestic animals, poultry, honey bees, silk worms and fishes to improve their taste preferences. The **trehalose** and saccharide compositions containing the same can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent and

stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of a drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The present **trehalose** and saccharide compositions containing the same can be used as a quality-improving agent and stabilizer for biologically active substances susceptible to loss of their effective ingredients and activities, as well as in health foods and pharmaceutical compositions containing biologically active substances. Examples of such a biologically active substance are lymphokines such as .alpha.-, .beta.- and .gamma.-interferons, tumor necrosis factor-.alpha. (TNF-.alpha.), tumor necrosis factor-.beta. (TNF-.beta.), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2 (IL-2); hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox vaccine, tetanus toxoid, Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. The present **trehalose** and saccharide compositions containing the same readily realize a preparation of the aforementioned biologically active substances into health foods and pharmaceutical compositions with a satisfactorily-high stability and quality without fear of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods to incorporate the present **trehalose** and saccharide compositions containing the same into the aforementioned substances and compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. The **trehalose** and saccharide compositions containing the same are usually incorporated into the aforementioned substances and compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
A liquid nutrient culture medium, consisting of 0.1 w/v % peptone, 0.1 w/v % yeasts extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium phosphate, 0.02 w/v % magnesium sulfate, 0.02 w/v % potassium chloride and water, was prepared. About 100 ml aliquots of the nutrient culture medium were placed in 500-ml Erlenmeyer flasks, autoclaved at 120.degree. C. for 20 minutes to effect sterilization, cooled and adjusted to pH 3.0 by the addition of sulphate, and then inoculated with a stock culture of **Sulfolobus acidocaldarius** ATCC 33909 and incubated at 75.degree. C. for 24 hours under stirring conditions of 130 rpm. The resultant cultures were pooled and used as a first seed culture. About 5 liter of a fresh preparation of the same nutrient culture medium as that used in the first seed culture was placed in a 10-liter fermenter, sterilized, cooled to 75.degree. C. and adjusted to pH 3.0, and then inoculated with one v/v % of the first seed culture and incubated at 75.degree. C. for about 48 hours while stirring under aerobic conditions at an aeration of 500 ml/min to obtain a second seed culture. About 250 liter of a fresh preparation of the same nutrient culture medium as that used in the first seed culture was placed in a 300-liter fermenter, sterilized, cooled to 75.degree. C. and adjusted to pH 3.0, and then inoculated with one v/v % of the second seed culture and incubated at 75.degree. C. for about 42 hours while stirring under aerobic conditions at an aeration of 100 ml/min. The present **trehalose**-releasing enzyme accumulated in the culture were respectively about 0.03 units/ml.

DEPR:

The objective thermostable **trehalose**-releasing enzyme and thermostable non-reducing saccharide-forming enzyme adsorbed on "DEAE-TOYOPEARL.RTM. were eluted from the column with 10 mM Tris-HCl buffer containing 0.1M sodium chloride. The resultant fractions were recovered.

DEPR:
The fractions thus obtained were dialyzed against a fresh preparation of 10 mM Tris-HCl buffer containing 1M ammonium sulfate. The dialyzed solutions thus obtained were centrifuged to remove insoluble substances, and the resultant supernatants were subjected to hydrophobic column chromatography using a column packed with 350 ml of "BUTYL-TOYOPEARL.RTM. 650", a hydrophobic gel commercialized by Tosoh Corporation, Tokyo, Japan. When material adsorbed on the gel was eluted from the column with a linear gradient buffer containing 1M to 0M ammonium sulfate, the thermostable **trehalose**-releasing enzyme and thermostable non-reducing saccharide-forming enzyme were eluted at different ammonium sulfate concentrations. The elution pattern of the column packed with "BUTYL-TOYOPEARL.RTM. was as shown in FIG. 1. The thermostable non-reducing saccharide-forming enzyme was eluted from the column at an ammonium sulfate concentration of about 0.2M, while the **trehalose**-releasing enzyme was eluted from the column at an ammonium sulfate concentration of about 0.2M. The fractions containing either of the objective enzymes were separately pooled and purified.

DEPR:
The objective thermostable **trehalose**-releasing enzyme was purified by subjecting the fractions eluted from a column packed with "BUTYL-TOYOPEARL.RTM." to gel filtration chromatography using "TOYOPEARL.RTM. HW-55" to recover fractions with the enzyme activity. The resultant fractions were subjected again to hydrophobic column chromatography using a column packed with "BUTYL-TOYOPEARL.RTM. 650", followed by subjecting to gel filtration chromatography using "SEPER ROSE 12HR 10/30" to recover fractions with the enzyme activity of the thermostable **trehalose**-releasing enzyme.

DEPR:
The enzyme activity, specific activity and yield of the thermostable non-reducing saccharide-forming enzyme in each purification step are as shown in Table 1, while those of the present thermostable **trehalose**-releasing enzyme are as shown in Table 2.

DEPR:
A portion of a purified thermostable **trehalose**-releasing enzyme preparation, obtained by the method in Experiment 2, was subjected to electrophoresis using a gel containing 10% sodium dodecylsulfate polyacrylamide, and determined its molecular weight to be about 54,000-64,000 daltons by making a comparison with marker proteins commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan.

DEPR:
To an aqueous solution containing 20% maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose as a substrate was added 2 units/g substrate, d.s.b., of a purified enzyme preparation obtained by the method in Experiment 4-1, and the resultant mixture was subjected to an enzymatic reaction at 40.degree. C. and pH 7.0 for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, filtered, decolored, desalted and concentrated to obtain a concentrated saccharide solution which was then subjected to ion-exchange column chromatography using "XT-1016 (Nat.sup.+ -form, polymerization degree of 4%)", an ion-exchanger commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan. In the column chromatography, the ion-exchanger was packed in 3-jacketed stainless-steel columns, having an inner diameter of 2.0 cm and a length of one m, which were then cascaded in series, heated to give the inner column temperature of 55.degree. C., applied with 5 v/v % of the concentrated saccharide solution against the resin while maintaining the temperature at 55.degree. C., and fed with 55.degree. C. hot water at SV (space velocity) of 0.13 to obtain the fractions of high-purity non-reducing saccharides having a **trehalose** structure as an end unit and having a degree of polymerization of 3 or higher. Among the resultant fractions, the purity of non-reducing saccharides in its high-purity preparation was 95.0% or higher, d.s.b. The fractions thus obtained were collected and the solution was dissolved in 0.1N sodium hydroxide, and heated at 100.degree. C. for 2 hours

to decompose the remaining reducing saccharides. The resultant solution was decolored with activated charcoal and purified with ion-exchanger (H.sup.+ and OH.sup.- form) to obtain the preparations rich in non-reducing saccharide preparations, and the purities of .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose in their high-purity preparations were respectively 99.0% or higher, d.s.b.

DEPR:
An aqueous solution containing 5%, d.s.b., of each one of the above five non-reducing saccharide preparations obtained by the method in Experiment 4-2, was mixed with 2 units/g substrate, d.s.b., of the purified **trehalose**-releasing enzyme obtained in Experiment 2, and subjected to an enzymatic reaction at 60.degree. C. and pH 5.5 for 48 hours. Each resultant reaction mixture was desalted and analyzed its composition on high-performance liquid chromatography (HPLC) using "WAKOBEADS WB-T-330", a column of Wako Pure Chemical Industries Ltd., Tokyo, Japan. As a control, a fresh preparation of the same enzyme was allowed to act on maltooligosaccharides such as maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. The resultant reaction mixture was analyzed its composition on HPLC. The results were in Table 3.

DEPR:
From these results, it is confirmed that the thermostable **trehalose**-releasing enzyme according to the present invention is a novel enzyme which has a mechanism of specifically hydrolyzing the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher to release **trehalose** from the non-reducing saccharide.

DEPR:
The reducing partial starch hydrolysates thus obtained or maltotriose having a degree of glucose polymerization of 3 as a substrate was dissolved in 10 mM phosphate buffer (pH 7.0) into a one % solution which was then mixed with 4 units/g substrate, d.s.b., of a purified non-reducing saccharide-forming enzyme and a purified **trehalose**-releasing enzyme prepared by the method in Experiment 2, and subjected to an enzymatic reaction at 40.degree. C. for 24 hours. After completion of the enzymatic reaction, a portion of resultant reaction mixture was desalted and analyzed on HPLC to identify its composition. Each remaining each reaction mixture was heated to 50.degree. C., adjusted to pH 4.5, admixed with 50 units/g substrate, d.s.b., of a glucoamylase specimen commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, and subjected to an enzymatic reaction for 10 hours. Similarly as above, a portion of each resultant reaction mixture was desalted and analyzed on HPLC to analyze its composition. The results were as shown in Table 4.

DEPR:
As is shown in Table 4, in the case of using as a substrate maltotriose having a degree of glucose polymerization of 3, the **trehalose** yield after enzymatic reaction using a thermostable non-reducing saccharide-forming enzyme and the present thermostable **trehalose**-releasing enzyme was relatively low, i.e. 2.2%, while in the case of using as a substrate partial starch hydrolysates having a degree of glucose polymerization of 10.8-36.8, the **trehalose** yield was relatively high. i.e. 63.3-81.2%. It was found that the higher the degree of glucose polymerization of reducing partial starch hydrolysates as a material, the higher the purity of the resultant **trehalose**. It was also found that the purity of the resultant **trehalose** can be more increased by allowing glucoamylase to act on the reaction mixture prepared by the above two enzymes, to decompose the concomitant non-reducing saccharides, having a **trehalose** structure as an end unit and

DEPR:
A nutrient culture medium was prepared, inoculated with microorganisms, and incubated for 42 hours in a fermenter by the same method in Experiment 1 except that **Sulfolobus acidocaldarius** (ATCC 49426), **Sulfolobus** solfataricus (ATCC 35091) and **Sulfolobus** solfataricus (ATCC 35092) were used as microorganisms in place of **Sulfolobus acidocaldarius** (ATCC 33909). According to the methods in Experiment 2, the cells were recovered from about 170 L of each resultant

culture, disrupted with ultrasonic to obtain a supernatant. The resultant supernatant was salted out with ammonium sulfate, dialyzed, subjected to an ion-exchange column and hydrophobic column chromatography to obtain a partially purified enzyme preparation, and followed by studying its properties. The results were in Table 5 together with those obtained in the case of using **Sulfolobus acidocaldarius** (ATCC 33909).

DEPR:
According to the method in Experiment 4-3, **trehalose** was prepared by using these partially purified enzyme preparations, and studied on its structure to find that, similarly as the thermostable **trehalose**-releasing enzyme from **Sulfolobus acidocaldarius** (ATCC 33909), every enzyme preparation released **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present thermostable **trehalose**-releasing enzyme, **trehalose** by using said enzyme, and saccharides containing the same; and Examples B illustrate compositions incorporating **trehalose** and saccharides containing the same.

DEPR:
A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was incubated by a fermenter for about 42 hours in accordance with the method in Experiment 1. After completion of the incubation, the resultant culture was concentrated with an SF-membrane to obtain about 5 L of cell suspension. The resultant suspension was treated with "MINI-LAB", a superhigh-pressure cell homogenizer, commercialized by Dainippon Pharmaceutical Co., to disrupt the cells. The resultant solution was centrifuged to recover about 4.8 L of supernatant. To the resultant supernatant was added ammonium sulfate to give a supersaturation degree of about 0.7, and the resultant solution was salted out and centrifuged to obtain a precipitate. The precipitate was dissolved in 10 mM tris-hydrochloride acid buffer (pH 8.5), and dialyzed against a fresh preparation of the same hydrochloride acid buffer. The resultant dialyzed solution was subjected five times to an ion-exchange column chromatography using a column packed with about 2 L of "SEPABEADS FP-DA13" which was equilibrated with said hydrochloride acid buffer, a gel commercialized by Mitsubishi Chemical Industries Ltd., Tokyo, Japan. The objective enzyme adsorbed on the ion exchanger was eluted from the column with a linear gradient buffer supplemented 0M to 0.5M sodium chloride, followed by recovering fractions with enzyme activity which was eluted from the column at about 0.15M sodium chloride. The resultant fractions were concentrated with an SF-membrane, and followed by recovering about 300 ml concentrated enzyme solution containing 32.6 units/ml of thermostable non-reducing saccharide-forming enzyme and 58.5 units/ml of thermostable **trehalose**-releasing enzyme. The fractions with enzyme activity thus recovered were dialyzed against a fresh preparation of 10 mM Tris-HCl buffer containing 1M ammonium sulfate, and the dialyzed solution thus obtained was centrifuged to remove insoluble substances. The resultant supernatant was subjected five time to hydrophobic column chromatography using a column packed with 350 ml of "BUTYL-TOYOPEARL.RTM. 650", a hydrophobic gel commercialized by Tosoh Corporation, Tokyo, Japan, and followed by separating thermostable non-reducing saccharide-forming enzyme and thermostable **trehalose**-releasing enzyme. To the suspension of potato starch having a concentration of 15 w/v % was added calcium carbonate to give a final concentration of 0.1 w/w %, adjusted to pH 6.0, admixed with "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, to give a concentration of 0.2 w/w % per g starch and subjected to an enzymatic reaction at 95.degree. C. for 15 min. The resultant mixture was autoclaved for 30 min (2 kg/cm.sup.2), cooled to 58.degree. C., adjusted to pH 5.5, admixed with 2,000 units/g starch of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 0.5 units/g starch of the above thermostable non-reducing saccharide-forming enzyme and 0.5 units/g starch of the above thermostable **trehalose**-releasing enzyme, and subjected to an enzymatic reaction for 96 hours. The resultant mixture was kept at 97.degree. C. for 30 min, cooled and filtered. The resultant filtrate was in the usual manner decolored with an activated charcoal, and purified by desalting it with ion-exchange resins in H- and OH-form. The resultant solution was concentrated into a syrup with a

concentration of about 60 w/v % in a yield of about 93%, d.s.b. The product contains 71.2% trehalose, 3.0% glucosyltrehalose, 1.3% maltosyltrehalose, 2.9% glucose, 11.1% maltose, 8.5% maltotriose, 2.0% maltooligosaccharides including higher molecular than maltotetraose and inclusive, d.s.b. The product has a mild and high-quality sweetness, as well as an adequate viscosity and moisture-retaining ability, and these properties render it arbitrarily useful in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
A saccharide solution as a feed solution, obtained by the method in Example A-1, was fractionated by using a column packed with "XT-1016 (Na.sup.+ -form, polymerization degree of 4%)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries Ltd., Tokyo, Japan. The procedure was as follows: The resin was packed in 4-jacketed stainless steel columns having an inner diameter of 5.4 cm, and the columns were cascaded in series to give a total gel-bed depth of 20 m. The columns were heated to give the inner column temperature of 55.degree. C. and fed with 5 v/v % of the saccharide solution against the resin while keeping at the temperature, followed by feeding to the columns with 55.degree. C. hot water to fractionate the saccharide solution and to remove concomitant saccharides such as maltose and maltotriose, and recovering trehalose-rich fractions. The fractions thus obtained were pooled, purified, concentrated, dried in vacuo and pulverized to obtain a high trehalose content powder in a yield of about 57%, d.s.b. The content of trehalose in the product is about 97%, d.s.b., and the product has a mild and high-quality sweetness, and because of these it is arbitrarily used in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient, diluent and filler.

DEPR:
A high trehalose content fraction obtained by the method in Example A-2 was in a usual manner decolored with an activated charcoal, desalted with an ion-exchanger, and concentrated into an about 70% solution which was then placed in a crystallizer, admixed with about 2% hydrous crystalline trehalose as a seed crystal, and gradually cooled to obtain a massecuite with a crystallinity of about 45%. The massecuite was sprayed from a nozzle equipped at the top of a drying tower at a high pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air being sent from the top of the drying tower, and the resultant crystalline powder was collected on a metal wire netting conveyer provided on the basement of the drying tower, and gradually moved out of the drying tower while a stream of 45.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an ageing tower and aged for 10 hours to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline trehalose in a yield of about 90% against the material high trehalose content fraction, d.s.b. The product is substantially non-hygroscopic and handles easily, and these render it arbitrarily useful in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient, diluent and filler.

DEPR:
A high trehalose content fraction obtained by the method in Example A-2 was purified similarly as in Example A-3, and the resultant was placed in an evaporator, and boiled up in vacuo to obtain a syrup with a moisture content of about 3.0%. The resultant syrup was placed in a crystallizer, admixed with one % anhydrous crystalline trehalose against the dry weight of the syrup, and crystallized at 120.degree. C. for 5 min under stirring conditions, and the resultant mixture was placed in a plain aluminum-container and aged at 100.degree. C. for 6 hours to obtain a block. The resultant block was pulverized by a cutter and dried by a fluidized-bed drying to obtain a powdery anhydrous crystalline trehalose with a moisture content of about 0.3% in a yield of about 85% against the material high trehalose content fraction, d.s.b. The product can be arbitrarily used as a desiccant in food products, cosmetics and pharmaceuticals, as well as their materials and intermediates. The product can be also used as a white powdery sweetener in a variety of compositions such as food products, cosmetics and pharmaceuticals.

DEPR:
In accordance with the method in Example A-1, a seed culture of a mutant of **Sulfolobus acidocaldarius** (ATCC 33909) was incubated by a fermenter for about 42 hours. After completion of the incubation, the resultant cells were membrane filtered with an SF-membrane to recover an about 5 L filtrate which was treated with "MINI-LAB", a superhigh-pressure cell homogenizer, commercialized by Dainippon Pharmaceutical Co., to disrupt the cells. The resultant solution was centrifuged to recover about 4.8 L supernatant. To the resultant supernatant was added ammonium sulfate to give a supersaturation degree of about 0.7, and the resultant solution was salted out and centrifuged to obtain a precipitate. The precipitate was dissolved in 10 mM phosphate buffer (pH 6.5), and dialyzed against a fresh preparation of the same phosphate buffer to recover about 600 ml enzyme solution containing about 15 units/ml of thermostable non-reducing saccharide-forming enzyme and about 12 units/ml of thermostable **trehalose**-releasing enzyme, and followed by subjecting to a hydrophobic column chromatography to recover 5,850 units of thermostable non-reducing saccharide-forming enzyme and 3,960 units of thermostable **trehalose**-releasing enzyme. One part by weight of potato starch was admixed with 6 parts by weight of water and 0.01 part by weight of "NEO-SPITASE", .alpha.-amylase, commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan. The resultant mixture was stirred and adjusted to pH 6.2, which was gelatinized and liquidized at a temperature of 85.degree. to 90.degree. C. The resultant liquidized solution was heated at 120.degree. C. for 10 min to inactivate the remaining .alpha.-amylase, cooled to 60.degree. C., adjusted to pH 5.5, admixed with 500 units/g starch of "PROMOZINE", pullulanase commercialized by Novo Nordisk Bioindustry, Copenhagen, Denmark, one unit/g starch of the above thermostable non-reducing saccharide-forming enzyme and one unit/g starch of the above thermostable **trehalose**-releasing enzyme, and subjected to an enzymatic reaction for 72 hours. The resultant mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzymes, adjusted to 50.degree. C. and pH 5.0, admixed with 10 units/g starch of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., subjected to an enzymatic reaction for 24 hours, and heated to inactivate the enzyme. The resultant solution was, in a usual manner, decolored, desalted with ion-exchange resins and concentrated into a syrup with a concentration of about 60%. The saccharide solution thus obtained contained 79.5% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 95% **trehalose,** d.s.b., and it was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with about 2% hydrous crystallized **trehalose** as a seed crystal and gradually crystallized under stirring conditions. The resultant was placed in a plain plastic-vessel and allowed to stand at an ambient temperature for 3 days to form a block. The resultant block was then pulverized by a cutter to obtain a powdery hydrous crystalline **trehalose** in a yield of about 70% against the material starch, d.s.b. The product is substantially non-hygroscopic and handles easily, and these render it arbitrarily useful in a variety of compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient, diluent and filler.

DEPR:
In accordance with the method in Experiment 1, a seed culture of **Sulfolobus** solfatarius (ATCC 35091) was incubated by a fermenter for about 42 hours. After completion of the incubation, in accordance with the method in Example A-1, the resultant cells were subjected to an SF-membrane filtration and a cell disruption. The resultant supernatant was salted out with ammonium sulfate to obtain a precipitate. The precipitate was dialyzed and followed by subjecting to an ion-exchange column chromatography to recover fractions with enzyme activity. The fractions were concentrated with an UF-membrane and followed by recovering about 150 ml concentrated enzyme solution containing 26.4 units/ml of thermostable non-reducing saccharide-forming enzyme and 57.5 units/ml of thermostable **trehalose**-releasing enzyme. The enzyme solution was subjected to a hydrophobic column chromatography to recover 2,650 units of thermostable non-reducing saccharide-forming enzyme and 5,950 units of thermostable **trehalose**-releasing enzyme. The suspension of potato starch having a

concentration of 6% was gelatinized by heating, adjusted to pH 4.5 and 50.degree. C., admixed with 500 units/g starch of isoamylase, and subjected to an enzymatic reaction for 20 hours. The resultant mixture was adjusted to pH 6.5, autoclaved at 120.degree. C. for 10 min, cooled to 95.degree. C., admixed with 0.1 w/w % per g starch of "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, and subjected to an enzymatic reaction for 15 min. The reaction mixture was autoclaved at 130.degree. C. for 30 min, cooled to 65.degree. C., admixed with one unit/g starch of the above non-reducing saccharide-forming enzyme and one unit/g starch of the above **trehalose**-releasing enzyme, and subjected to an enzymatic reaction for 72 hours. The resultant mixture was kept at 97.degree. C. for 30 min, adjusted to pH 5.0 and 50.degree. C., admixed with 10 units/g starch of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., subjected to an enzymatic reaction for 24 hours, and heated to inactivate the enzyme. The resultant solution was, in a usual manner, decolored, desalted with ion-exchange resins and concentrated into a syrup with a concentration of about 60%. The saccharide solution thus obtained contained 80.9% **trehalose,** d.s.b. The saccharide solution was concentrated to give a concentration of about 84%, and then placed in a crystallizer, admixed with about 2% hydrous crystalize **trehalose** as a seed crystal and gradually crystallized under stirring conditions. The resultant was placed in a plain plastic-vessel and allowed to stand at an ambient temperature for 3 days to form a block. The resultant block was then pulverized by a cutter to obtain a powdery hydrous crystalline **trehalose** in a yield of about 90% against the material starch, d.s.b. The product is substantially non-hygroscopic and handles easily, and these render it arbitrarily useful in a variety of compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient, diluent and filler.

DEPR:
To one part by weight of a powdery hydrous crystalline **trehalose,** obtained by the method in Example A-3, were homogeneously added 0.01 part by weight of ".alpha.G SWEET", an .alpha.-glycosyl stevioside product commercialized by Toyo Sugar Refining Co., Ltd., Tokyo, Japan, and 0.01 part by weight of "ASPARTAME", an L-aspartyl-L-phenylalanine methylester product commercialized by Ajinomoto Co., Ltd., Tokyo, Japan, and the resultant mixture was fed to a granulator to obtain a granular sweetener. The product has a satisfactory sweetness and an about 2.5-fold higher sweetening power of sucrose, and the caloric value is about 1/2.5 of that of sucrose. The product having a satisfactory stability does not decompose other sweeteners to be mixed, and with which it is suitably used as a low-caloric sweetener for low-caloric food products directed to fat persons and diabetics who are restricted to a reduced calorie intake. The product substantially does not form insoluble glucans, and this renders it useful for sweetening food products to prevent dental caries.

DEPR:
One hundred parts by weight of 55% sucrose solution was mixed while heating with 30 parts by weight of a **trehalose** syrup, obtained by the method in Example A-1, and the resultant solution was concentrated in vacuo until the moisture content decreased to below 2%. The concentrated solution was admixed with one part by weight of citric acid and adequate amounts of a lemon flavor and a coloring agent, and the resultant mixture was in a usual manner formed into the desired product. The product is a high-quality hard candy having a satisfactory taste and biting property, as well as having no fear of causing crystallization of sucrose.

DEPR:
Three parts by weight of a gum base was melted by heating until it softened, and the resultant was mixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method in Example A-3, and further mixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was in usual manner kneaded by a roll, formed and packed to obtain the desired product. The product is a chewing gum having a satisfactory texture and taste.

DEPR:
Three parts by weight of a **trehalose** syrup obtained by the method in Example A-1 and one part by weight of sucrose were dissolved in 100 parts by weight of

fresh milk, and the resultant solution was sterilized by heating with a plate heater, and condensed to give a concentration of 70%, followed by aseptically canning the resultant concentrate into the desired product. The product has a mild sweetness and a satisfactory taste, and these render it arbitrarily useful as a seasoning for baby foods, foods for infants, fruit, coffee, cocoa and tea.

DEPR:
One hundred and seventy-five parts by weight of defatted milk, 130 parts by weight of a **trehalose** syrup prepared by the method in Example A-1, and 50 parts by weight of a high lactosucrose content powder disclosed in Japanese Patent Laid-Open No. 281,795/92 were dissolved in 1,150 parts by weight of water, and the resultant solution was sterilized by heating at 65.degree. C. for 30 min, cooled to 40.degree. C., admixed in usual manner with 30 parts by weight of lactic acid bacteria as a starter, and incubated at 37.degree. C. for 8 hours to obtain a beverage containing lactic acid bacteria. The product is a beverage containing lactic acid bacteria with a satisfactory taste and flavor. The product containing oligosaccharides stably retains lactic acid bacteria and promotes the growth of bifid bacteria.

DEPR:
Thirty-three parts by weight of a powdered orange juice prepared by spray drying was mixed to homogeneity with 50 parts by weight of a high **trehalose** content powder obtained by the method in Example A-2, 10 parts by weight of sucrose, 0.65 parts by weight of anhydrous citric acid, 0.1 part by weight of malic acid, 0.1 part by weight of L-ascorbic acid, 0.1 part by weight of sodium citrate, 0.5 parts by weight of pullulan, and an adequate amount of a powdered flavor. The resultant mixture was pulverized, fed to a fluidized-bed granulator and sprayed with a **trehalose** syrup as a binder obtained by the method in Example A-1 while sending to the contents 40.degree. C. air at a flow rate of 150 m.sup.3. The granules thus obtained were weighed and packaged to obtain the desired product. The product containing 30% orange juice, d.s.b., retains its high quality for a relatively-long period of time without giving an unsatisfactory taste and smell.

DEPR:
One hundred parts by weight of corn starch, 100 parts by weight of a **trehalose** syrup obtained by the method in Example A-1, 80 parts by weight of maltose, 20 parts by weight of sucrose, and one part by weight of salt were mixed to homogeneity. The resultant mixture was admixed with 280 parts by weight of egg, and gradually added with 1,000 parts by weight of a boiling milk. The mixture thus obtained was continuously stirred while heated, and the heating was stopped when the corn starch in the mixture was completely gelatinized to render the whole contents semitransparent, followed by cooling the mixture and adding thereto an adequate amount of a vanilla flavor. The resultant mixture was weighed, injected and packaged to obtain the desired product. The product has a smooth surface and gloss, as well as a mild taste and sweetness.

DEPR:
Ninety parts by weight of rice powder was admixed to homogeneity with 20 parts by weight of corn starch, 40 parts by weight of sucrose, 80 parts by weight of a powder containing hydrous crystalline **trehalose** obtained by the method in Example A-3 and 4 parts by weight of pullulan to obtain "uiro-no-moto". The "uiro-no-moto" was kneaded with appropriate amounts of "maccha (a green tea powder)" and water and the resultant mixture was divided in vessels and steamed for 60 minutes to obtain "maccha-uiro" The product has a smooth gloss, good palatability and delicious taste, and also has a long shelf life because retrogradation of starch effectively suppressed.

DEPR:
Ten parts by weight of "adzuki" beans as a material was boiled by the addition of water in usual manner, followed by removing the astringency and harshness of the beans, as well as water-soluble impurities, to obtain about 21 kg "adzuki-tsubu-an". To the resultant was added 14 parts by weight of sucrose, 5 parts by weight of a **trehalose** syrup obtained by the method in Example A-1, and 4 parts by weight of water, and the resultant mixture was boiled, admixed with a small amount of salad oil, and carefully kneaded up so as not to paste the beans. Thus, the desired product was obtained in a yield of about 35 kg. The product free from discoloration induced by boiling has a satisfactory taste and

flavor, and these render it useful as a material "an" for bean-jam buns, buns with bean-jam filling, dumplings, bean-jam-filled wafers, sherbets and ice creams.

DEPR:
One hundred parts by weight of wheat powder, 2 parts by weight of yeast, 5 parts by weight of sugar, one part by weight of a powder containing **trehalose** obtained by the method in Example A-2, 0.1 part by weight of inorganic yeast food were kneaded with water in usual manner to effect fermentation at 26.degree. C. for 2 hours, and further aged for 30 min, followed by baking the resultant. The product is a high-quality bread having a satisfactory hue and rising, as well as a satisfiable elasticity and mild sweetness.

DEPR:
To one thousand parts by weight of ham meat slices was added and ground to homogeneity 15 parts by weight of salt and 3 parts by weight of potassium nitrate, and the resultant slices were piled up and allowed to stand overnight in a cold-storage room. Thereafter, the resultant slices were first soaked for 7 days in a cold-storage room in a salt solution consisting of 500 parts by weight of water, 100 parts by weight of salt, 3 parts by weight of potassium nitrate, 40 parts by weight of a powdery hydrous crystalline **trehalose** prepared by the method in Example A-6, and an adequate amount of a peppermint, then washed with cold water in usual manner, tied up, smoked, cooked, cooled and packaged to obtain the desired product. The product is a high-quality ham having a satisfactory hue, taste and flavor.

DEPR:
One part by weight of 40% "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was admixed with 2 parts by weight of a powdery hydrous crystalline **trehalose** prepared by the method in Example A-6, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfactory taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:
To one part by weight of "akamiso" (a kind of miso) was added 3 parts by weight of a powdery anhydrous crystalline **trehalose** obtained by the method in Example A-4, and the mixture was poured into a metal plate having hemisphere wells on its surface and allowed to stand at an ambient temperature overnight to obtain "miso" solids, about 4 g weight each, which were then subjected to a pulverizer to obtain the desired product. The product can be arbitrarily used as a seasoning for instant noodles and soups, as well as a "miso" confectionery.

DEPR:
Egg yolks prepared from fresh eggs were sterilized at 60.degree.-64.degree. C. by a plate heater, and the resultant liquid was admixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method in Example A-4 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was allowed to convert into hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk. The product can be arbitrarily used as a material for confectioneries for premixes, sherbets, ice cream and emulsifiers, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings. The product can be also used as a skin refiner and hair restorer.

DEPR:
Two parts by weight of polyoxyethylene glycol monostearate, 5 parts by weight of glyceryl monostearate, self-emulsifying, 2 parts by weight of a high **trehalose** content powder obtained by the method in Example A-2, one part by weight of .alpha.-glycosyl rutin, one part by weight of liquid petrolatum, 10 parts by weight of glyceryl tri-2-ethylhexanoate, and an adequate amount of an antiseptic were in usual manner dissolved by heating. The resultant solution was admixed with 2 parts by weight of L-lactic acid, 5 parts by weight of

1,3-butylene glycol and 66 parts by weight of refined water, and the resultant mixture was emulsified by a homogenizer and admixed with an adequate amount of a flavor under stirring conditions to obtain a cosmetic cream. The product exhibits an antioxidant activity and has a relatively-high stability, and these render it arbitrarily useful as a high-quality sunscreen, skin-refining agent and skin-whitening agent.

DEPR:
A half part by weight of ginseng extract was mixed with 1.5 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method in Example A-4, and the resultant mixture was transferred to a plain container, allowed to stand for 2 days to convert anhydrous crystalline **trehalose** into hydrous crystalline **trehalose** to form a block. The resultant block was pulverized by a cutter and classified to obtain a powdery ginseng extract. The product and adequate amounts of powdery vitamins B1 and B2 were subjected to a granulator to obtain a powdery ginseng extract containing vitamins. The product thus obtained can be arbitrarily used as a tonic, fatigue-relieving agent and vitality-imparting agent. The product can be also used as a hair restorer.

DEPR:
A natural human interferon-.alpha. preparation, commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was in usual manner fed to a column of an immobilized anti-human interferon-.alpha. antibody to adsorb the natural human interferon-.alpha., and a buffer containing calf serum albumin as a stabilizer was fed to the column, followed by removing an excessive amount of the albumin. Thereafter, the interferon-.alpha. was eluted from the column with a physiological saline containing 5% powder rich in **trehalose,** prepared by the method in Example A-2, while the pH of the physiological saline was varying. The resultant eluate was membrane filtered, and the filtrate was dehydrated by the addition of about 20-fold volumes of "FINETOSE.RTM.", an anhydrous crystalline maltose powder commercialized by Hayashibara Shoji, Inc., Okayama, Japan, followed by pulverizing the resultant dehydrated product, and tabletting the resultant powder by a tabletting machine to obtain tablets containing about 150 units of the natural human interferon-.alpha. per one tablet, about 200 mg weight. The product can be orally administered as a sublingual tablet to patients at a dose of 1-10 tablets/adult/day, and arbitrarily used to treat vital diseases, allergies, rheumatisms, diabetes and malignant tumors. More particularly, the product can be suitably used as a therapeutic agent for AIDS and hepatitis, the number of patients suffering from these diseases has been remarkably increased. The **trehalose** and maltose incorporated in the product act as a stabilizer for the natural human interferon-.alpha., so that the activity is well retained for a relatively-long period of time even at an ambient temperature.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method in Example A-3, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose,** one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to obtain a sugar coated tablet having a satisfactory gloss and appearance. The product has a relatively-high shock tolerance and retains its high quality for a relatively-long period of time.

DEPR:
A composition consisting of 500 parts by weight of a powdered hydrous crystalline **trehalose** obtained by the method in Example A-3, 270 parts by weight of dried yolk, 209 parts by weight of defatted milk, 4.4 parts by weight of sodium chloride, 1.8 parts by weight of potassium chloride, 4 parts by weight of magnesium sulfate, 0.01 part by weight of thiamine, 0.1 part by weight of sodium ascorbate, 0.6 parts by weight of vitamin E acetate and 0.04 parts by weight of nicotine amide was prepared, and the composition was divided into 25 g aliquot in small moistureproof laminated aluminum packs which were then heat-sealed. One pack of the product is dissolved in about 150-300 ml water and the resultant solution is usable as an a liquid supplemental

nutrition parenterally administrable to the nasal cavity, stomach or intestine.

DEPR:
Two hundred parts by weight of powdered hydrous crystalline **trehalose** obtained by the method in Example A-3 and 300 parts by weight of maltose were admixed with 50 parts by weight of methanol containing 3 parts by weight of iodine, and the resultant was mixed with 200 parts by weight of 10 w/v % pullulan to obtain a traumatic ointment which has an appropriate extensity and adhesiveness. The product shortens a therapeutic period and cures traumas without a scar by reason that the iodine incorporated in the product exhibits sterilizing effects and also the **trehalose** incorporated in the product supplements nutrition into traumas.

DEPR:
As is evident from above, the present novel thermostable **trehalose**-releasing enzyme releases **trehalose** from non-reducing saccharides having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher, is superior in thermal stability, and forms **trehalose** in a relatively-high yield when acted on reducing partial starch hydrolysates together with a thermostable non-reducing saccharide-forming enzyme. The **trehalose** thus obtained can be readily separated and purified, and the resultant purified **trehalose** and saccharide compositions containing the same have a satisfactory stability as well as a relatively-high quality and moderate sweetness. Since **trehalose** can be readily assimilated and absorbed by living bodies when orally taken, the product can be used as energy source, and the product per se and saccharide compositions containing the same can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient, diluent and filler in a variety of compositions such as food products, cosmetics and pharmaceuticals.

DEPR:
Thus, the present invention provides a novel technique to prepare **trehalose** and saccharide compositions containing the same in an industrial-scale and relatively-low cost from partial starch hydrolysates prepared from starch, a cheap and abundant natural source. Therefore, the present invention gives an unfathomable great influence on the fields such as starch-, enzyme- and biochemical-sciences, and other industrial fields, especially, food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomably great.

DEPL:
Properties of thermostable **trehalose**-releasing enzyme

DEPL:
Production of **trehalose** by thermostable **trehalose**-releasing enzyme

DEPL:
Preparation of non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher

DEPL:
Preparation of **trehalose** from non-reducing saccharides by thermostable **trehalose**-releasing enzyme

DEPL:
Preparation of **trehalose** from non-reducing partial starch hydrolysates

DEPL:
having a degree of glucose polymerization of 3 or higher, into **trehalose** and glucose molecules.

DEPL:
Preparation of thermostable **trehalose**-releasing enzyme from other microorganisms of the genus **Sulfolobus** and its properties

DEPV:
1. Thermostable **trehalose**-releasing enzyme of the present invention

specifically hydrolyzes the linkage between a **trehalose** moiety and a glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher to form **trehalose** and a non-reducing saccharide having a degree of glucose polymerization of one or more; and

DEPV:
2. Maltooligosaccharides are not hydrolyzed by the present **trehalose**-releasing enzyme.

DEPW:
Specifically hydrolysing the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and having a degree of glucose polymerization of 3 or higher;

DETL:
TABLE 3 ______________________________________ Elution time Percent- on HPLC age Substrate Product (min) (%) ______________________________________ Glucosyltrehalose **Trehalose** 27.4 7.2 Glucose 33.8 3.9 Glucosyltrehalose 23.3 88.9 Maltosyltrehalose **Trehalose** 27.4 40.2 Maltose 28.7 40.5 Maltosyltrehalose 21.6 19.3 Maltotriosyltrehalose **Trehalose** 27.4 41.1 Maltotriose 25.9 58.2 Maltotriosyltrehalose 19.7 0.7 Maltotetraosyltrehalose **Trehalose** 27.4 34.0 Maltotetraose 24.1 65.8 Maltotetraosyltrehalose 18.7 0.2 Maltopentaosyltrehalose **Trehalose** 27.4 29.1 Maltotetraose 22.6 70.6 Maltotetraosyltrehalose 17.8 0.3 Maltotriose Maltotriose 25.9 100 Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

____

DETL:
TABLE 4 ______________________________________ Degree of glucose polymerization of Composition reducing partial (%) starch hydrolysate Reaction product A* B** ______________________________________ 36.8 **Trehalose** 81.2 82.5 Glucose 1.2 17.5 Reducing oligosaccharides 13.6 0.0 .alpha.-glycosyltrehalose 4.0 0.0 27.5 **Trehalose** 79.5 81.8 Glucose 1.8 18.2 Reducing oligosaccharides 14.1 0.0 .alpha.-glycosyltrehalose 4.6 0.0 19.8 **Trehalose** 77.3 80.2 Glucose 2.2 19.8 Reducing oligosaccharides 15.3 0.0 .alpha.-glycosyltrehalose 5.2 0.0 16.5 **Trehalose** 73.4 77.5 Glucose 2.5 22.5 Reducing oligosaccharides 18.1 0.0 .alpha.-glycosyltrehalose 6.0 0.0 10.8 **Trehalose** 63.3 68.5 Glucose 5.7 31.5 Reducing oligosaccharides 22.8 0.0 .alpha.-glycosyltrehalose 8.2 0.0 3 **Trehalose** 2.2 19.9 (Maltotriose) Glucose 10.4 80.1 Maltose 18.5 0.0 Maltotriose 42.0 0.0 .alpha.-glucosyltrehalose 26.9 0.0 ______________________________________
Note: The symbol "*" means a composition after enzymatic reaction of a nonreducing saccharideforming enzyme and the present trehalosereleasing enzyme, and the symbol "**" means a composition after enzymatic reaction of glucoamylase. In the Table, the wording ".alpha.-glycosyltrehalose" means nonreducing saccharides having a **trehalose** structure as an end unit and having a degree of glucose polymerization degree of 3 or higher.

DETL:
TABLE 5 ______________________________________ Enzyme activity in eluate from ion- Optimum exchange temper- Thermal column ature Optimum stability pH Microorganism (unit) (.degree.C.) pH (.degree.C.) stability ______________________________________ **Sulfolobus** 2470 About About Up to About **acidocaldarius** 75.degree. C. 5.5-6.0 about 5.5-9.5 (ATCC 33909) 85.degree. C. **Sulfolobus** 1850 About About Up to About **acidocaldarius** 75.degree. C. 5.5-6.0 about 5.5-9.5 (ATCC 49426) 85.degree. C. **Sulfolobus** 1220 About About Up to About solfatarius 75.degree. C. 5.5-6.0 about 4.5-8.5 (ATCC 35091) 85.degree. C. **Sulfolobus** 445 About About Up to About solfatarius 75.degree. C. 5.5-6.0 about 4.5-8.5 (ATCC 35092) 85.degree. C.

____

DETL:
______________________________________ Composition: calcium hydrogen phosphate 45.0% pullulan 2.95% sodium laurate 1.5% glycerine 20.0% polyoxyethylene sorbitan laurate 0.5% antiseptic 0.05% powdery hydrous crystalline

trehalose, 12.0% obtained by the method in Example A-3 maltitol 5.0% water 13.0%

CLPR:
1. A purified **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and the remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 and which enzyme is capable of acting on the non-reducing saccharide at a temperature of over 55.degree. C. while retaining its activity.

CLPR:
5. The enzyme of claim 4, wherein said microorganism is a member selected from the group consisting of those of the genus **Sulfolobus** and mutants thereof.

CLPR:
8. The process of claim 7, wherein said microorganism is a member selected from the group consisting of those of the genus **Sulfolobus** and mutants thereof.

CLPR:
9. A process for preparing **trehalose** which contains a step of allowing the enzyme of claim 1 to act on a solution containing a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3.

CLPR:
11. The process of claim 9, wherein said **trehalose** is a member selected from the group consisting of hydrous crystalline **trehalose,** anhydrous crystalline **trehalose,** and mixture thereof.

CLPV:
(a) contacting a solution containing a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 with the enzyme of claim 1 to produce **trehalose**; and

CLPV:
(b) incorporating **trehalose** obtained in step (a) into at least one other saccharide.

CLPV:
(a) contacting a solution containing a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 with the enzyme of claim 1 to produce **trehalose**; and

CLPV:
(b) incorporating **trehalose** obtained in step (a) into a food material.

CLPV:
(a) contacting a solution containing a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 with the enzyme of claim 1 to produce **trehalose**; and

CLPV:
(b) incorporating **trehalose** obtained in step (a) into a cosmetically-acceptable carrier.

CLPV:
(a) contacting a solution containing a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3 with the enzyme of claim 1 to produce **trehalose**; and

CLPV:
(b) incorporating **trehalose** obtained in step (a) into a pharmaceutically-acceptable carrier.

CLPW:
Specifically hydrolyzing the linkage between a **trehalose** moiety and the

remaining glycosyl moiety in a non-reducing saccharide having a **trehalose** structure as an end unit and a degree of glucose polymerization of at least 3;

ORPL:
Lama, L. et al. "Starch Conversion With Immobilized Thermophilic Archaebacterium **Sulfolobus** Solfataricus" Biotech. Ltrs., vol. 12, No. 6, pp. 431-432 (1990).

ORPL:
Hoelzle, I. et al., "Increased Accumulation of **Trehalose** in Rhizobia Cultured Under 1% Oxygen", Appl. Environ. Micro., p. 3213-3215, (1990).

USPT

US-CL-CURRENT: 435/100,435/101 ,435/252.1 ,536/123.1 ,536/123.13

US-PAT-NO: 5716838
DOCUMENT-IDENTIFIER: US 5716838 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: February 10, 1998
INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/252.2,435/100 ,435/101 ,435/252.1 ,536/123.1 ,536/123.13

ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act-on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.
12 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

ABPL:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act-on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.

BSPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses, more particularly, to a novel non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, as well as to its preparation and microorganisms capable of producing said enzyme. The present invention further relates to a composition containing a non-reducing saccharide having a **trehalose** structure as an end unit which is preparable with said enzyme, a relatively-low reducing saccharide containing said non-reducing saccharide, and/or **trehalose** prepared from these saccharides.

BSPR:
Trehalose or .alpha., .alpha.**-trehalose** has long been known as a non-reducing saccharide consisting of glucose units. As described in Advances in Carbohydrate Chemistry, Vol.18, pp.201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), **trehalose** widely exists in microorganisms, mushrooms, insects, etc., though the content is relatively low. Since non-reducing saccharides including **trehalose** do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, non-reducing saccharides can be used with amino acids without causing browning and deterioration. Because of this, there has been in great demand to establish a method for preparation of such a non-reducing saccharide.

BSPR:
In conventional preparations of **trehalose,** as disclosed in Japanese Patent Laid-Open No.154,485/75, microorganisms are utilized, or as proposed in Japanese Patent Laid-Open No.216,695/83, maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** present in microorganisms as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, if specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, maltose as a substrate could not be used at a relatively-high concentration; (ii) Since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) it is substantially difficult to maintain the reaction systems stably and to continue their enzymatic reactions smoothly. Thus, there has not yet been realized an industrial-scale preparation of **trehalose**.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No.88, pp.67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been reported to be scientifically almost impossible in this field." Thus, an enzymatic preparation of **trehalose** by using starch as a material has been deemed to be scientifically very difficult.

BSPR:
In order to attain the aforementioned object, the present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme, which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates.

BSPR:
As a result, we isolated novel microorganisms of the genera Rhizobium, named as "Rhizobium sp. M-11", and Arthrobacter, named as "Arthrobacter sp. Q36", from the respective soils in Okayama-city, Okayama, Japan, and in Soja-city, Okayama, Japan; and found that the microorganisms produce a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and that the objective non-reducing saccharides are readily prepared when the enzyme is allowed to act on reducing partial starch hydrolysates.

BSPR:
We also found that **trehalose** can be prepared by first allowing the enzyme to act on reducing partial starch hydrolysates, then subjecting the resultant non-reducing saccharides to the action of glucoamylase or .alpha.-glucosidase. Thus, the present inventors accomplished this invention. Also, we extensively screened microorganisms capable of producing the enzyme from conventional microorganisms.

BSPR:
As a result, it was found that microorganisms of the genera Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium and Terrabacter produce the present non-reducing saccharide-forming enzyme as the microorganisms of the genera Rhizobium and Arthrobacter, and we accomplished this invention. Also, we established preparations of compositions such as food products, cosmetics and pharmaceuticals which contain the present non-reducing saccharides, relatively-low reducing saccharides containing the non-reducing saccharides and/or **trehalose** prepared from these saccharides, and accomplished this invention.

DEPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses. The present invention further relates to

a microorganism capable of producing said enzyme, non-reducing saccharides prepared with said enzyme, relatively-low reducing saccharides containing said non-reducing saccharides, **trehalose** prepared from these saccharides, and compositions containing either or both of these saccharides and **trehalose**.

DEPR:
The present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and eventually found the objective microorganisms.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol.1 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Rhizobium. The microorganism is similar to those of the species Rhizobium meliloti in some properties, but they are distinguishable with the fact that the present microorganism utilizes maltose, lactose and mannitol but forms no acid, and it produces a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such a microorganism having these properties.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol.2 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Arthrobacter. The microorganism is characterized by producing a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such an enzyme.

DEPR:
The concentration of the reducing partial starch hydrolysates used as a substrate in the invention is not specifically restricted. While the present enzymatic reaction proceeds even with a 0.1% solution of a substrate, the enzymatic reaction more favorably proceeds with solutions having a concentration of 2% or higher, preferably, those having a concentration of 5-50% of a substrate, d.s.b. Under these concentrations non-reducing saccharides having a **trehalose** structure are readily formed in a satisfactorily-high yield. Suspensions containing insoluble substrates can be used in the invention. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 55.degree. C., preferably, a temperature in the range of 40.degree.-50.degree. C. The reaction pH used in the present enzymatic reaction is controlled in the range of 5-10, preferably, in the range of about 6-8. The reaction time used in the present enzymatic reaction is adequately chosen dependently on the conditions of the enzymatic reaction.

DEPR:
If necessary, the present non-reducing saccharides having a **trehalose** structure or relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase and .alpha.-glucosidase in order to control their sweetness and reducing power or to lower their viscosity; and the resultant products can be further treated such that the remaining reducing saccharides are hydrogenated into sugar alcohols to diminish their reducing powder.

DEPR:
More particularly, **trehalose** is readily prepared by allowing glucoamylase or .alpha.-glucosidase to act on the present non-reducing saccharides or relatively-low reducing saccharides containing them. A high **trehalose** content fraction is obtained by allowing glucoamylase or .alpha.-glucosidase to act on these saccharides to form a mixture of **trehalose** and glucose, and subjecting the mixture to the aforementioned purifications such as ion-exchange column chromatography to remove glucose. The high **trehalose** content fraction can be

arbitrary purified and concentrated into a syrupy product, and, if necessary, the syrupy product can be concentrated into a supersaturated solution, followed by crystallizing hydrous- or anhydrous-crystalline **trehalose** and recovering the resultant crystal.

DEPR:
In order to prepare hydrous crystalline **trehalose,** an about 65-90% solution of **trehalose** with a purity of about 60% or higher is placed in a crystallizer, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10.degree.-90.degree. C., to obtain a massecuite containing hydrous crystalline **trehalose**. .Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the invention to prepare from the massecuite hydrous crystalline **trehalose** or crystalline saccharides containing it.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline **trehalose** from the mother liquor, and, if necessary the hydrous crystalline **trehalose** is washed by spraying with a small amount of cold water to facilitate the preparation of hydrous crystalline **trehalose** with an increased purity. In the case of spray drying, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by spraying massecuites with a concentration of 70-85%, d.s.b., and a crystallinity of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60.degree.-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto air heated to about 30.degree.-60.degree. C. In the case of block pulverization, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallinity of about 10-60%, to stand for about 0.1-3 days in order to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

DEPR:
Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into the anhydrous form, it is generally prepared by providing a concentrated solution of **trehalose** with a moisture content less than 10%; placing the solution in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50.degree.-160.degree. C., preferably, a temperature in the range of 80.degree.-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present non-reducing saccharides are hydrolyzed by amylases such as .alpha.-amylase derived from pancreas into relatively-low molecular weight non-reducing oligosaccharides or maltooligosaccharides, and these oligosaccharides are readily hydrolyzed by .alpha.-glucosidase and intestinal enzymes into glucose and **trehalose** molecules. The resultant **trehalose** is readily hydrolyzed by trehalase into glucoses. Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** can be utilized as an energy source by the body when orally administered. These present saccharides and **trehalose** are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** have a satisfiable stability and sweetness, and those in crystalline form can be arbitrarily used as a sugar coating material for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinylpyrrolidone. These saccharides and **trehalose** have properties such as osmotic pressure-controlling ability, filler-imparting ability, gloss-imparting ability, moisture-retaining ability,

viscosity-imparting ability, substantial no fermentability, ability to prevent retrogradation of gelatinized starch, and ability to prevent crystallization of other saccharides.

DEPR:
Anhydrous crystalline **trehalose** can be arbitrarily used as a desiccant for food products, cosmetics, pharmaceuticals, and their materials and intermediates, and can be readily formed into compositions in the form of powder, granule and tablet with a satisfactory stability and quality.

DEPR:
Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient and desiccant in a variety of compositions such as food products, tobaccos, cigarettes, feeds, pet foods, cosmetics and pharmaceuticals.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, isomaltooligosaccharide, galactooligosaccharide, fructooligosaccharide, lactosucrose, sorbitol, maltitol, lactitol, dihydrocharcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as a powdery or crystalline **trehalose** prepared from these saccharides, can be used intact, or, if necessary they can be mixed with an excipient, filler and binder and formed into granules, spheres, shot-rods, plates, cubes and tablets, prior to their use.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides have the following features: (i) They have a sweetness which well harmonizes with other materials having sour-, acid-, salty-, bitter-, astringent- and delicious-tastes; and (ii) they are highly acid- and heat-resistant. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in seasonings such as amino acids, peptides, soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake), "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare" (a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), nucleic acid condiments, mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
Also, the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be freely used for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker), "arare-mochi" (a rice/cake cube), "okoshi" (a millet-and-rice "uiro" (a sweet rice jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly,

pao de Castella and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as Jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickles and pickled products such as "fukujin-zuke" (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish ham, fish sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tenpura" (a Japanese deep-fat fried fish paste); "chinmi" (relish) such as "uni-no-shiokara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle); "saki-surume" (dried squid strips) and "fugu-no-mirin-boshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk products; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liquors; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix and "sokuseki-shiruco" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and beverages such as baby foods, foods for therapy, beverages supplemented with nutrition, peptide foods and frozen foods; as well as for improving the tastes and qualities of the aforementioned food-products.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be also used in feeds and pet foods for animals such as domestic animals, poultry, honey bees, silk worms and fishes in order to improve their taste preferences. These saccharides and **trehalose** can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent and stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used as a quality-improving agent and stabilizer in biologically active substances which may contain unstable effective ingredients and activities, as well as in health foods and pharmaceuticals containing the biologically active substances. Examples of such a biologically active substance are lymphokines such as .alpha.-, .beta.- and .gamma.-interferons, tumor necrosis factor-.alpha. (TNF-.alpha.), tumor necrosis factor-.beta. (TNF-.beta.), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2; hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox vaccine, tetanus toxoid, Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase. and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. By using the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides, the aforementioned biologically active substances are arbitrarily prepared into health foods and pharmaceuticals with a satisfactorily-high stability and quality without a fear

of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods for incorporating the present non-reducing saccharides, relatively-low reducing saccharides containing them and/or **trehalose** prepared from these saccharides into the above-mentioned compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. These saccharides and **trehalose** are usually incorporated into the above-mentioned compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
Fifty mg aliquots of non-reducing saccharides P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for 6 hours. The only saccharides detected in every resultant mixture on HPLC analysis were glucose and **trehalose**. The contents of the detected glucose and **trehalose,** and their molecular ratios were as shown in Table 5.

DEPR:
As evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one mole of glucose and one mole of **trehalose**; P II, hydrolyzed into two moles of glucose and one mole of **trehalose**; (iii) PIII, hydrolyzed into three moles of glucose and one mole of **trehalose**; (iv) P IV, hydrolyzed into four moles of glucose and one mole of **trehalose**; and (v) P V, hydrolyzed into five moles of glucose and one mole of **trehalose**.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more moles of glucose bound to one mole of **trehalose** via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing saccharide having a degree of glucose polymerization of 3 (DP 3) and consisting of one mole of glucose bound to one mole of **trehalose**; P II, a non-reducing saccharide having DP 4 and consisting of two moles of glucose bound to one mole of **trehalose**; P III, a non-reducing saccharide having DP 5 and consisting of three moles of glucose bound to one mole of **trehalose**; P IV, a non-reducing saccharide having DP 6 and consisting of four moles of glucose bound to one mole of **trehalose**; and P V, a non-reducing saccharide having DP 7 and consisting of five moles of glucose bound to one mole of **trehalose**. It was revealed that, when .beta.-amylase was act on these non-reducing saccharides similarly as with glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one mole of maltose and one mole of P I, one mole of maltose and one mole of P II, and two moles of maltose and one mole of P I.

DEPR:
Based on these results, it was concluded that the enzymatic reaction of the present non-reducing saccharide-forming enzyme is an intramolecular reaction without changing the molecular weights of the substrates used, i.e. an intramolecular reaction without changing their degrees of glucose polymerization. It was concluded that the non-reducing saccharides P I, P II, PIII, P IV and P V were the respective .alpha.-glycosyl trehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.**-trehalose** residue) of .alpha.-glucosyl **trehalose,** .alpha.-maltosyl **trehalose,** .alpha.-maltotriosyl **trehalose,** .alpha.-maltotetraosyl **trehalose** and .alpha.-maltopentaosyl **trehalose**.

DEPR:
As evident from the results in Tables 7 and 8, it was revealed that similarly as in Experiment 6 with glucoamylase the saccharide preparations P I, P II, PIII, P IV and P V were hydrolyzed by .alpha.-glucosidase and rat intestinal acetone powder into glucose and **trehalose** molecules.

DEPR:
To the resultant hydrolysate obtained with .alpha.-glucosidase or rat intestinal acetone powder was added one unit trehalase derived from pig kidney, an enzyme preparation of Sigma Chemical Company, St., Louis, USA, and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC to reveal that **trehalose,** formed from the saccharide preparations P I, P II, PIII, P IV and P V, was hydrolyzed by trehalase into glucose molecules.

DEPR:
Based on these results, it was concluded that the present non-reducing saccharide-forming enzyme is a novel enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates to a non-reducing end unit, a **trehalose** residue, i.e. a **trehalose** structure.

DEPR:
By using the purified enzyme preparation obtained in Experiment 10, the preparation and the confirmation of the structure of non-reducing saccharides were conducted in accordance with the methods in Experiments 4 and 6. As a result, it was revealed that the enzyme preparation forms one or more non-reducing saccharides, which saccharide has a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher, when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
In accordance with the method in Experiment 12, non-reducing saccharides were prepared by using partially purified enzyme preparations from these known microorganisms, and their structures were studied to find that, similarly as the non-reducing saccharide-forming enzyme from Rhizobium sp. M-11, every enzyme preparation formed non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose**; and Examples B illustrate compositions containing one or more of these saccharides and **trehalose**.

DEPR:
The saccharide solution thus obtained contained 29.5% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 90% **trehalose,** d.s.b.. The fraction was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with about 2%, d.s.b., hydrous crystalline **trehalose** as a seed crystal and gradually cooled to obtain a massecuite with a degree of crystallization of about 45%. The massecuite was sprayed from a nozzle equipped on the top of a spraying tower at a pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air sent from the top of the spraying tower, and the resultant crystalline powder was collected on a metal wire netting conveyer provided on the basement of the spraying tower, and gradually moved out of the tower while a stream of 40.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an ageing tower and aged for 10 hours to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline **trehalose**.

DEPR:
In accordance with the method in Example A-3, 30% suspension of corn starch was subjected to the action of an .alpha.-amylase specimen commercialized by Novo Industri A/S, Copenhagen, Denmark; a maltotetraose forming amylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan; and an .alpha.-amylase specimen commercialized by Ueda Chemical Co., Ltd.,

Osaka, Japan. The resultant mixture was autoclaved at 120.degree. C., cooled to 45.degree. C., admixed with 2 units per g starch of a non-reducing saccharide-forming enzyme prepared by the above-mentioned method, and subjected to an enzymatic reaction for 64 hours. The reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme. In accordance with the method in Example A-6, the resultant solution was subjected to the action of glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, decolored, desalted and concentrated into an about 60% solution. The saccharide solution thus obtained contained about 25% **trehalose,** d.s.b. The saccharide solution was fractionated on column chromatography using a strongly-acidic cation-exchange resin to obtain fractions rich in **trehalose**. The fractions were pooled, placed in a vessel and boiled down under a reduced pressure into a syrup with a moisture content of about 4.0%. The syrup was placed in a crystallizer and admixed with one % of anhydrous crystalline **trehalose,** as a seed crystal, with respect to the syrup, d.s.b., followed by crystallizing anhydrous crystalline **trehalose** at 95.degree. C. for 5 min while stirring. The resultant was transferred to an aluminum container and aged at 100.degree. C. for 6 hours to form a block. The resultant block was pulverized by a cutting machine and subjected to a fluidized-bed drying to obtain a powdery anhydrous crystalline **trehalose** with a moisture content of about 0.3%.

DEPR:
Three parts by weight of gum base was melted by heating until it softened, and the resultant was mixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method of Example A-6, and further mixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was kneaded by a roll in the usual manner, formed and packed to obtain the desired product.

DEPR:
Forty % "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was mixed with 2 parts by weight of a powder containing hydrous crystalline **trehalose** prepared by the method of Example A-6, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfactory taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:
Egg yolks prepared from fresh eggs were sterilized at 60.degree.-64.degree. C. by a plate heater, and the resultant liquid was mixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method of Example A-8 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was permitted to hydrate to hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method of Example A-6, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose,** one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to, obtain a sugar coated tablet having a satisfiable gloss and appearance.

DEPR:
As evident from above, the present novel non-reducing saccharide-forming enzyme converts reducing partial starch hydrolysates into non-reducing saccharides in a satisfactorily-high yield under a relatively-mild enzymatic reaction condition without changing the degrees of glucose polymerization of the reducing partial starch hydrolysates. The non-reducing saccharides, which can

be readily separated and purified, and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, have a satisfactory stability, quality and mild sweetness. These products are assimilated and utilized as an energy source by the body when orally administered. These non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be arbitrarily used in compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thus, the present invention provides a novel technique to prepare in an industrial-scale and at a relatively-low cost non-reducing saccharides, which could not have been readily obtained in spite of their great demands, by using reducing partial starch hydrolysates prepared from starch as a cheap and abundant source, as well as to prepare relatively-low reducing saccharides containing the non-reducing saccharides, and **trehalose** prepared from these saccharides. The present invention has a great influence on the fields such as starch-, enzyme- and biochemical-sciences; and other industrial fields, especially, food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomable.

DEPV:
Forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

DEPV:
(1) The present non-reducing saccharide-forming enzyme forms non-reducing saccharides having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher without changing their degrees of glucose polymerization; and

DEPV:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is hydrolyzed by glucoamylase into one mole of **trehalose** and two moles of glucose.

DETL:
Carbon source Utilization Acid formation D-Glucose + + D-Galactose + + D-Fructose + + L-Arabinose + + D-Xylose + + L-Rhamnose + + Maltose + - Sucrose + + Lactose + - **Trehalose** + - Raffinose + + Mannitol + - Dextrin + - Dulcitol + -

DETL:
TABLE 5 Saccharide Glucose **Trehalose** Molecular ratio preparation (%) (%) (Glucose/Trehalose) P I 36.2 63.8 1.07 P II 52.0 48.0 2.06 P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11

DETL:
TABLE 7 Saccharide composition of hydrolysate with .alpha.-glucosidase Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) P I 36.5 63.0 0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3 0.3

DETL:
TABLE 8 Saccharide composition of hydrolysate with rat intestinal acetone powder Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) P I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P V 73.4 26.5 0.1

CLPR:
1. A biologically pure culture of a microorganism which produces an enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on a reducing partial starch hydrolysate, which is a microorganism selected from the group consisting of Rhizobium sp. M-11 (FERM BP-4130) and its mutants.

CLPR:
2. A biologically pure culture of a microorganism which produces an enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on a reducing partial starch hydrolysate, which is a microorganism selected from the group consisting of Arthrobacter sp. Q36 (FERM BP-4316) and its mutants.

CLPR:
3. A method for decreasing the reducing power of a reducing partial starch hydrolysate comprising contacting a solution containing a reducing partial starch hydrolysate with an enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on a reducing partial starch hydrolysate but not on **trehalose**.

CLPR:
6. A process for producing **trehalose** which comprises:

CLPR:
7. The process of claim 6, wherein the step (b) further includes a step of crystallizing **trehalose**.

CLPR:
8. The process of claim 7, wherein said **trehalose** is hydrous- or anhydrous-crystalline **trehalose**.

CLPR:
9. The process of claim 6, wherein the resultant mixture in the step (b) is further subjected to column chromatography using a strongly-acidic cation-exchange resin to increase the content of **trehalose**.

CLPR:
10. The process of claim 6, wherein the **trehalose** structure in said non-reducing saccharide is located in its end unit.

CLPR:
11. The process of claim 10, wherein said non-reducing saccharide is an .alpha.-glycosyl **trehalose** shown by the formula:

CLPV:
(a) contacting a solution containing a reducing partial starch hydrolysate with an enzyme to form a non-reducing saccharide having a **trehalose** structure, said enzyme acting on said reducing partial starch hydrolysate but not on **trehalose**;

CLPV:
(b) contacting the product of step (a) with glucoamylase or .alpha.-glucosidase to form **trehalose**; and

CLPV:
(c) recovering the resultant **trehalose**.

CLPV:
and the symbol "T" means .alpha., .alpha.-**trehalose** residue.

ORPL:
Hoelzle et al., "Increased Accumulation of **Trehalose** in Rhizobia Cultured under 1% Oxygen," Applied and Environmental Microbiology, pp. 3213-3215, Oct. 1990.

ORPL:
Journal of the Chemical Society, May 1965 Letchworth, GB, pp. 3489-3490, Birch, "method of obtaining crystalline anhydrous alphaalpha-**trehalose**".

ORPL:
Biotechnology Letters, vol. 12, No. 6, Jun. 1990, pp. 431-432; Lama et al; "Starch conversion with immobilized thermophilic Archaebacterium **Sulfolobus** Solfataricus".

USPT

US-CL-CURRENT: 435/100,435/101 ,435/200 ,514/53 ,514/54 ,514/60 ,536/123.13 ,536/124

US-PAT-NO: 5714368
DOCUMENT-IDENTIFIER: US 5714368 A
TITLE: Thermostable non-reducing saccharide-forming enzyme its production and uses
DATE-ISSUED: February 3, 1998
INVENTOR-INFORMATION:

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ABSTRACT:
Disclosed are novel thermostable non-reducing saccharides-forming enzyme, its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as **Sulfolobus acidocaldarius** (ATCC 33909 and ATCC 49426) and **Sulfolobus** solfataricus (ATCC 35091 and ATCC 35092), and capable of forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates at a temperature of over 55.degree. C. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.
23 Claims, 4 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 4

ABPL:
Disclosed are novel thermostable non-reducing saccharides-forming enzyme, its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as **Sulfolobus acidocaldarius** (ATCC 33909 and ATCC 49426) and **Sulfolobus** solfataricus (ATCC 35091 and ATCC 35092), and capable of forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates at a temperature of over 55.degree. C. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals.

BSPR:
The present invention relates to a novel thermostable non-reducing saccharide-forming enzyme, and its preparation and uses, more particularly, to a novel thermostable non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, and to its preparation. The present invention further relates to a non-reducing saccharide having a **trehalose** structure as an end unit which is preparable by utilizing the thermostable non-reducing saccharide-forming enzyme and to a relatively-low reducing saccharide containing the non-reducing saccharide, as well as to a **trehalose** prepared from these saccharides and to a composition containing these non-reducing saccharides.

BSPR:
Trehalose or .alpha., .alpha.-**trehalose** is known as a non-reducing saccharide consisting of glucose units. As described in Advance in Carbohydrate Chemistry, Vol.18, pp.201-225 (1963), published by Academic Press, USA, and in Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), **trehalose** widely exists in microorganisms, mushrooms, insects, etc., through

the content is relatively low. Since **trehalose** is a non-reducing saccharides, it does neither induce the amino-carbonyl reaction with the substances containing amino groups such as amino acids and proteins nor alter amino acid-containing substances. Thus, **trehalose** is expected to be used without fear of causing an unsatisfiable browning and deterioration. Because of these, it has been in great demand to establish an industrial-scale preparation of **trehalose**.

BSPR:
In conventional preparation of **trehalose,** as disclosed in Japanese Patent Laid-Open No.154,485/75, microorganisms are utilized, or as proposed in Japanese Patent Laid-Open No.216,695/83, maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** present in microorganisms as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, unless specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has not yet been realized as an industrial-scale preparation because of the following demerits: (i) maltose as a substrate could not be used at a relatively-high concentration because **trehalose** is formed via glucose phosphate; (ii) the yield of the objective **trehalose** is relatively low; (iii) it is substantially difficult to continue the enzymatic reactions smoothly while retaining their reaction systems stably.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", pp.67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been reported to be scientifically almost impossible in this field." Thus, an enzymatic preparation of **trehalose** using starch as a material has been deemed to be scientifically difficult.

BSPR:
Considering the aforementioned circumstances, the present inventors have energetically studied enzymes which are capable of forming saccharides having a **trehalose** structure when allowed to act on starch hydrolysates. As a result, the present inventors found that microorganisms of the genus Rhizobium, named as "Rhizobium sp. M-11", and microorganisms of the genus Arthrobacter, named as "Arthrobacter sp. Q36", isolated respectively from the soils as disclosed in Japanese Patent Application No.349,216/93, are capable of producing novel non-reducing saccharide-forming enzymes which form non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, and found that **trehalose** is readily preparable by allowing glucoamylase or .alpha.-glucosidase to act on the non-reducing saccharides having a **trehalose** structure as an end unit obtainable by this novel enzymes.

BSPR:
Enzymes derived from the microorganisms of the genus Rhizobium or the genus Arthrobacter, however, are relatively-low in thermal stability. Thus, in case that these enzymes are utilized for preparing non-reducing saccharides having a **trehalose** structure as an end unit and **trehalose,** it is necessary to allow the enzymes to act on at a temperature of below 55.degree. C. With regard to the temperature of enzymatic reaction, as described in the column titled "Enzymes related to saccharides" in the chapter titled "Enzymes related to saccharides and their applications" in "Koso-Ouyou-no-Chishiki" (Knowledge on Enzyme Applications), the first edition, pp.80-129 (1986) that "In the conditions of industrial-scale enzymatic reactions for saccharification, the reactions at a temperature of below 55.degree. C. involves a risk of contamination and a decrease of pH during the reaction", in long-time enzymatic reactions using starch as a material, when an enzyme is allowed to act on at a temperature of below 55.degree. C., because of contamination and a decrease of pH of reaction mixtures which may inactivate the activity of such enzymes, and it is necessary to add lysozyme for the prevention of contamination and the pH control of the reaction mixtures. In addition, when the hydrolysis of partial starch

hydrolysates is relatively low, insoluble substances may be formed due to retrogradation of starch. On the other hand, since a thermostable enzyme can act on at a relatively-high temperature, contamination during the enzymatic reaction is less concerned and the retrogradation of partial starch hydrolysates is hardly caused. Thus, it has been in great demand to establish novel preparations of non-reducing saccharides, having a **trehalose** structure as an end unit, and **trehalose** from such non-reducing saccharides by utilizing a thermostable non-reducing saccharide-forming enzyme capable of acting on at a temperature of over 55.degree. C.

BSPR:
To attain the aforementioned object, the present inventors have extensively screened microorganisms capable of producing a novel thermostable non-reducing saccharide-forming enzyme, which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. As a result, the present inventors found that microorganisms of the genus **Sulfolobus,** named as "**Sulfolobus acidocaldarius**" ATCC 33909 and ATCC 49426, and as "**Sulfolobus** solfataricus" ATCC 35091 and ATCC 35092, produce a novel thermostable non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates and also is stable up to a temperature of about 85.degree. C., and found that the non-reducing saccharide can be readily prepared at the objective temperature of over 55.degree. C. when the thermostable enzyme is allowed to act on reducing partial starch hydrolysates. The present inventors also found that **trehalose** is readily preparable by first allowing the thermostable enzyme to act on reducing partial starch hydrolysates, then subjecting the resultant non-reducing saccharides to the action of glucoamylase or .alpha.-glucosidase. Thus, the present inventors accomplished this invention. Also, the present inventors established preparations of compositions such as food products, cosmetics and pharmaceuticals which contain the present non-reducing saccharides, relatively-low reducing saccharides containing the same and/or **trehalose,** and accomplished this invention.

DEPR:
The present invention relates to a novel thermostable non-reducing saccharide-forming enzyme, and its preparation and uses. The present invention further relates to a microorganism capable of producing said enzyme, non-reducing saccharides prepared with said enzyme, relatively-low reducing saccharides containing said non-reducing saccharides, **trehalose** prepared from these saccharides, and compositions containing either or both of these non-reducing saccharides and **trehalose**.

DEPR:
The present inventors have extensively screened microorganism capable of producing a novel thermostable non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and eventually found the objective microorganisms.

DEPR:
In the present specification, unless specified otherwise, a novel thermostable non-reducing saccharide-forming enzyme, which forms a non-reducing saccharide having a **trehalose** structure as an end unit when allowed to act on reducing partial starch hydrolysates and acts on at a temperature of over 55.degree. C., is referred to as a thermostable non-reducing saccharide-forming enzyme.

DEPR:
Now, the present inventors found that microorganisms of the genus **Sulfolobus,** named as "**Sulfolobus acidocaldarius**" ATCC 33909 and ATCC 49426, and as "**Sulfolobus** solfataricus" ATCC 35091 and ATCC 35092, are capable of producing a novel thermostable non-reducing saccharide-forming enzyme.

DEPR:
In addition to the above-mentioned microorganisms, other strains of the genus **Sulfolobus** and their mutants can be suitably used in the present invention as long as they produce the present thermostable non-reducing saccharide-forming enzyme which forms the non-reducing saccharide having a **trehalose** structure as

an end unit when allowed to act on reducing partial starch hydrolysates.

DEPR:
The concentration of the reducing partial starch hydrolysates used as a substrate in the invention is not specifically restricted. While the present enzymatic reaction proceeds even with a 0.1% solution of a substrate, the enzymatic reaction more favorably proceeds with solutions having a concentration of 2% or higher, preferably, those having a concentration of 5-50% of a substrate, d.s.b. Under these concentrations non-reducing saccharides having a trehalose structure are readily formed in a satisfactorily-high yield. The reaction temperature used in the present invention enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 85.degree. C., preferably, a temperature in the range of 55.degree.-70.degree. C. The reaction pH used in the present enzymatic reaction is controlled to in the range of 3-9, preferably, in the range of about 4-7. The reaction time used in the present enzymatic reaction is adequately chosen depending on the conditions of the enzymatic reaction, generally, in the range of 0.1-100 hours in the case of using enzyme in an amount of about 0.1-100 units/g substrate, d.s.b.

DEPR:
If necessary, the present non-reducing saccharides having a trehalose structure thus obtained or relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase and .alpha.-glucosidase to control their sweetness and reducing power or to lower their viscosity; and the resultant products can be further treated with processings of hydrogenating the remaining reducing saccharides into sugar alcohols to diminish their reducing power.

DEPR:
More particularly, trehalose is readily prepared by allowing glucoamylase or .alpha.-glucosidase to act on the present non-reducing saccharides or relatively-low reducing saccharides containing them. A high trehalose content fraction is obtained by allowing glucoamylase or .alpha.-glucosidase to act on these saccharides to form a mixture of trehalose and glucose, and subjecting the mixture to the aforementioned purification such as ion-exchange column chromatography to remove glucose. The high trehalose content fraction can be arbitrarily purified and concentrated into a syrupy product, and, if necessary, the syrupy product can be further concentrated into a supersaturated solution, followed by crystallizing it into hydrous- or anhydrous-crystalline trehalose and recovering the resultant crystal.

DEPR:
To prepare hydrous crystalline trehalose, an about 65-90% solution of trehalose with a purity of about 60% or higher, d.s.b., is placed in a crystallizer, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10.degree.-90.degree. C., to obtain a massecuite containing hydrous crystalline trehalose. Also, the continuous crystallization to prepare hydrous crystalline trehalose while concentrating a solution of trehalose under reduced pressure can be favorably used in the present invention. Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the present invention to prepare from the massecuite hydrous crystalline trehalose or crystalline saccharides containing it.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline trehalose from the mother liquor, and, if necessary, the hydrous crystalline trehalose is washed by spraying thereto with a small amount of cold water to facilitate the preparation of hydrous crystalline trehalose with an increased purity. In the case of spray drying, crystalline saccharides with no or substantially free of hygroscopicity are readily prepared by spraying massecuites with a concentration of 60-85%, d.s.b., and a crystallization percentage of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60.degree.-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto a 30.degree.-60.degree. C. hot air. In the case of block

pulverization, crystalline saccharides with no or substantially free of hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallization percentage of about 10-60%, d.s.b., to stand for a period from about several hours to 3 days to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

DEPR:
Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into anhydrous one, it is generally prepared by providing a high **trehalose** content solution with a moisture content less than 10%; placing the solution in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50.degree.-160.degree. C., preferably, a temperature in the range of 80.degree.-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present non-reducing saccharides are hydrolyzed by amylases such as .alpha.-amylase derived from pancreas into relatively-low molecular weight non-reducing oligosaccharides or maltooligosaccharides, and these oligosaccharides are readily hydrolyzed by .alpha.-glucosidase and intestinal enzymes into glucose and **trehalose** molecules. The resultant **trehalose** is readily hydrolyzed by trehalase into glucose. Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose**, can be utilized as an energy source by the body when orally administered. These present saccharides and **trehalose** are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener. These saccharides and **trehalose** have properties such as osmotic pressure-controlling ability, filler-imparting ability, gloss-imparting ability, moisture-retaining ability, viscosity-imparting ability, ability to prevent crystallization of other saccharides, substantial no fermentability, and ability to present retrogradation of gelatinized starch.

DEPR:
The present **trehalose** can be utilized parenterally as a liquid feeding and infusion without fear of toxicity and side effect, preferably, utilized as an energy source by the body. Also, the present **trehalose** has a satisfiable stability and sweetness, and those in crystalline form can be arbitrarily used as a sugar coating material for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinylpyrrolidone.

DEPR:
Anhydrous crystalline **trehalose** can be arbitrarily used as a desiccant for food products, cosmetics, pharmaceuticals, and their materials and intermediates, and readily formed into compositions in the form of powder, granule and tablet with a satisfactory stability and quality.

DEPR:
Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient and desiccant in a variety of compositions such as food products, tobaccos, cigarettes, feeds, pet foods, cosmetics and pharmaceuticals.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, isomaltooligosaccharide, galactooligosaccharide, fructooligosaccharide, lactosucrose, sorbitol, maltitol, lactitol, dihydrocharcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl

L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as a powdery or crystalline **trehalose** prepared from these saccharides, can be used intact, or, if necessary they can be admixed with an excipient, filler and/or binder and formed into granules, spheres, short-rods, plates, cubes and tablets, prior to their use.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides have a sweetness which well harmonizes with other materials having sourness, acidness, saltines, astringency, deliciousness and bitterness, and they are highly acid- and heat-resistant. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in seasonings such as amino acids, peptides, soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake)", "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare" (a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), nucleic acid condiments, mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
Also, the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be favorably used for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker)", "arare" (a glutinous rice cracker), "okoshi" (a millet-and-rice cracker), "mochi" (a rice paste)"manju" (a bun with a bean-jam), "uiro" (a sweet rice jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly, pao de Castellan and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickled and pickled products such as "fukujin-zuke (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish meat ham, fish meat sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tenpura" (a Japanese deep-fat fried fish pastefoods)"; "chinmi" (relish) such as "uni-no-shiokara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle), "saki-surume" (dried squid strips) and "fugu-no-mirinboshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk products; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liqueurs; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix, "sokuseki-shiruko" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and

beverages such as baby foods, foods for therapy, beverages supplemented with nutrition, peptide foods and frozen foods; as well as for improving the taste and qualities of the aforementioned food-products.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in feeds and pet foods for animals such as domestic animals and poultry, honey bee, silkworm and fish to improve their taste preference. These saccharides and **trehalose** can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent and stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used as a quality-improving agent and stabilizer in biologically active substances susceptible to deterioration of their effective ingredients and activities, as well as in health foods and pharmaceuticals containing biologically active substances. Examples of such a biologically active substances are lymphokines such as interferon-.alpha., interferon-.beta., interferon-.gamma., tumor necrosis factor-.alpha., tumor necrosis factor-.beta., macrophage-migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2; hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox vaccine, tetanus toxoid, Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; and viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. By using the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides, the aforementioned biologically active substances are arbitrarily prepared into health foods and pharmaceuticals with a satisfactorily-high stability and quality without fear of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods to incorporate the present non-reducing saccharides, relatively-low reducing saccharides containing them and/or **trehalose** prepared from these saccharides into the above-mentioned compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. These saccharides and **trehalose** are usually incorporated into the above-mentioned compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
A liquid nutrient culture medium, consisting of 0.1 w/v % peptone, 0.1 w/v % yeasts extract, 0.2 w/v % ammonium sulfate, 0.05 w/v % potassium phosphate, 0.02 w/v % magnesium sulfate, 0.02 w/v % potassium chloride and water, was prepared. About 100 ml aliquots of the nutrient culture medium were placed in 500-ml Erlenmeyer flasks, autoclaved at 120.degree. C. for 20 minutes to effect sterilization, cooled and adjusted to pH 3.0 by the addition of sulphate, and then inoculated with a stock culture of **Sulfolobus acidocaldarius** ATCC 33909 and incubated at 70.degree. C. for 24 hours under stirring conditions of 130 rpm. The resultant cultures were pooled and used as a first seed culture.

DEPR:
As evident from the results in Table 1, it was revealed that the present purified enzyme formed non-reducing saccharides having a **trehalose** structure as

an end unit which were composed of .alpha.-glucosyl **trehalose** to .alpha.-maltopentaosyl **trehalose** when allowed to act on partial starch hydrolysates having a degree of glucose polymerization of 3 or higher which were composed of maltotriose to maltoheptaose. Also, it was revealed that in addition to the remaining substrate and non-reducing saccharides producible without altering the degree of glucose polymerization, a relatively-small amount of glucose as a hydrolysate of substrate and lower molecular weight maltooligosaccharides as well as non-reducing saccharides prepared from them existed in the reaction mixture, and the present purified enzyme had a slight hydrolytic activity to the non-reducing saccharide-forming activity. The yields of non-reducing saccharides, prepared by allowing the present purified enzyme to act on the substrates, and reducing saccharides prepared from hydrolysates were respectively 30.2% and 27.6% in the case of using maltotriose as a substrate, 65.4% and 18.4% for maltotetraose, about 74-75% and 2-3% for maltopentaose and maltoheptaose having a degree of glucose polymerization 4 to 5, and it was revealed that non-reducing saccharides were formed at a relatively-high yield from maltooligosaccharides having a degree of glucose polymerization of 5 or higher, and also hydrolysates were formed in small quantity. Also, it was revealed that no saccharide was newly formed from glucose and maltose.

DEPR:
A nutrient culture medium was prepared, inoculated with microorganisms, and incubated for 42 hours in a fermenter by the same method in Experiment 1 except that **Sulfolobus acidocaldarius** (ATCC 49426), **Sulfolobus** solfataricus (ATCC 35091) and **Sulfolobus** solfataricus (ATCC 35092) were used as microorganisms in place of **Sulfolobus acidocaldarius** (ATCC 33909). The cells were recovered from about 170 liters of each resultant culture, disrupted with ultrasonic to obtain a supernatant, and the resultant supernatant was salted out with ammonium sulfate, dialyzed, and subjected to an ion-exchange column and hydrophobic column chromatography to obtain a partially purified enzyme preparation, followed by studying its properties. The results were in Table 2.

DEPR:
In accordance with the method in Experiment 2-1, non-reducing saccharides were prepared by using these partially purified enzyme preparations, and studied on their structures to find that, similarly as the thermostable non-reducing saccharide-forming enzyme from **Sulfolobus acidocaldarius** (ATCC 33909), every enzyme preparation formed non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher when allowed to act on reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose**; and Examples B illustrate compositions containing one or more of these saccharides and **trehalose**.

DEPR:
A seed culture of **Sulfolobus acidocaldarius** (ATCC 33909) was inoculated in a nutrient culture medium and incubated by a fermenter for about 42 hours in accordance with the method in Experiment 1. After completion of the incubation, the resultant culture was concentrated with an SF-membrane and centrifuged to recover cells. The cells thus obtained were disrupted with ultrasonic, and from the suspension an supernatant was prepared, salted out with ammonium sulfate, dialyzed, and subjected to an ion-exchange column and hydrophobic column chromatography to obtain an enzyme solution containing 18.0 units/ml of a partially purified enzyme preparation having a specific activity of about 20 units/mg protein. The suspension of potato starch having a concentration of 6 w/v % was gelatinized by heating, adjusted to pH 4.5 and 50.degree. C., admixed with 2,500 units/g starch of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and subjected to an enzymatic reaction for 20 hours. The resultant mixture was adjusted to pH 6.5, autoclaved at 120.degree. C. for 10 min, cooled to 60.degree. C., admixed with 30 units/g starch of "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, and subjected to an enzymatic reaction for 24 hours. The reaction mixture was autoclaved at

120.degree. C. for 20 min, cooled to 65.degree. C., adjusted to pH 5.5, admixed with one unit/g starch of the above thermostable non-reducing saccharide-forming enzyme, and subjected to an enzymatic reaction for 96 hours. The resultant mixture was kept at 97.degree. C. for 30 min, cooled and filtered. The resultant filtrate was in usual manner decolored with an activated charcoal, and purified by desalting it with ion-exchange resins in H- and OH-form. The resultant solution was concentrated into a syrup with a concentration of about 70 w/v % in a yield of about 90%. The product exhibits a DE 24.6, and contains as a non-reducing saccharide 12.0% .alpha.-glucosyl **trehalose,** 5.5% .alpha.-maltosyl **trehalose,** 29.9% .alpha.-maltotriosyl **trehalose,** 1.5% maltotetraosyl **trehalose** and 2.2% .alpha.-maltopentaosyl **trehalose,** d.s.b. The product has a mild and high-quality sweetness, as well as an adequate viscosity and moisture-retaining ability, and these properties render it arbitrarily useful in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
A saccharide solution as a feed solution, obtained by the method in Example A-1, was fractionated on a column packed with "XT-1016 (polymerization degree of 4%, Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Tokyo Organic Chemical Industries Ltd., Tokyo, Japan to increase the content of non-reducing sccharides. The procedure was as follows: The resin was packed in 4 jacketed-stainless steel columns having an inner diameter of 5.4 cm, and the columns were cascaded in series to give a total gel-bed depth of 20 m. The columns were heated to give the inner column temperature of 55.degree. C., and fed with 5 v/v % of the saccharide solution against the resin while keeping at the temperature, and the saccharide solution was fractionated by feeding to the columns with 55.degree. C. hot water at SV 0.13 to elute fractions rich in glucose and maltose, followed by recovering fractions rich in non-reducing saccharides. The fractions rich in non-reducing saccharides were pooled, purified, concentrated, dried in vacuo, and pulverized to obtain a powdery product containing non-reducing saccharides in a yield of about 64%, d.s.b. The product exhibits a DE 4.8, and contains as a non-reducing saccharide 18.2% .alpha.-glucosyl **trehalose,** 7.9% .alpha.-maltosyl **trehalose,** 46.6% .alpha.-maltotriosyl **trehalose,** 2.3% maltotetraosyl **trehalose** and 3.4% .alpha.-maltopentaosyl **trehalose,** d.s.b. Similarly as the product in Example A-1, the product has a mild and high-quality sweetness, as well as an adequate viscosity and moisture-retaining ability, and these properties render it arbitrarily useful in food product, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thirty-three % suspension of corn starch, d.s.b., was admixed with calcium carbonate to give the final concentration of 0.1%, d.s.b., and the resultant mixture was adjusted to pH 6.5, admixed with 0.2%, d.s.b., per g starch of "TERMAMYL 60 L", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, and subjected to an enzymatic reaction at 95.degree. C. for 15 min. The resultant mixture was autoclaved at 120.degree. C. for 10 min, cooled to 55.degree. C., admixed with 5 units/g starch of maltotetraose-forming amylase as disclosed in Japanese Patent Laid-Open No.240,783/63, commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and subjected to an enzymatic reaction for 6 hours. The resultant mixture was admixed with 30 units/g starch of ".alpha.-amylase 2A", .alpha.-amylase commercialized by Ueda Chemical Co., Ltd., Osaka, Japan, and subjected to an enzymatic reaction at 65.degree. C. for 4 hours. The resultant mixture was autoclaved at 120.degree. C. for 10 min, cooled to 45.degree. C., adjusted to pH 5.5, admixed with 2 units/g starch of a thermostable non-reducing saccharide-forming enzyme obtained by the method in Example A-1, and subjected to an enzymatic reaction for 48 hours. The resultant mixture was kept at 97.degree. C. for 30 min, cooled and filtered to obtain a filtrate which was then decolored with an activated charcoal in usual manner, and purified by desalting it with ion-exchange resins in H- and OH-form, followed by concentrating the resultant solution to obtain a 70% syrup in a yield of about 90%, d.s.b. The product exhibits a DE 17.1, and contains as a non-reducing saccharide 8.9% .alpha.-glucosyl **trehalose,** 29.3% .alpha.-maltosyl **trehalose,** 0.8% .alpha.-maltotriosyl **trehalose,** 0.7%

maltotetraosyl **trehalose** and 0.7% .alpha.-maltopentaosyl **trehalose,** d.s.b. The product has a mild and high-quality sweetness, as well as an adequate viscosity and moisture-retaining ability, and these properties render it arbitrarily useful in food product, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
A saccharide solution as a feed solution, obtained by the method in Example A-3, was column chromatographed in accordance with the method in Example A-2 except that "50W-X4 (Mg.sup.++ -form)", a strongly-acidic cation exchange resin commercialized by Dow Chemical Co., Midland, Mich., USA, was used as a resin for fractionation to increase the content of .alpha.-maltosyl **trehalose** and to obtain a .alpha.-maltosyl **trehalose** rich fraction. The fraction was purified, concentrated and spray dried to obtain a powdery product rich in non-reducing saccharides in a yield of about 41%, d.s.b. The product contains as a non-reducing saccharide 10.9% .alpha.-glucosyl **trehalose,** 61.3% .alpha.-maltosyl **trehalose** and 1.0% .alpha.-maltotriosyl **trehalose,** exhibits a DE 2.5, and have a relatively-low reducibility. Similarly as the product in Example A-3, the product has a mild and high-quality sweetness, as well as an adequate viscosity and moisture-retaining ability, and these properties render it arbitrarily useful in food product, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Forty parts by weight of "PINE-DEX #4", a partial starch hydrolysate commercialized by Matsutani Chemical Ind., Tokyo, Japan, was dissolved in 60 parts by weight of water, and the resultant solution was heated to 65.degree. C., adjusted to pH 5.5, admixed with one unit/g partial starch hydrolysate of a thermostable non-reducing saccharide-forming enzyme prepared by the method in Example A-1, and subjected to an enzymatic reaction for 96 hours. Thereafter, the reaction mixture was heated at 97.degree. C. for 30 min to inactivate the remaining enzyme, diluted to give a concentration of about 20%, d.s.b., admixed with 10 units/g partial starch hydrolysate of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 10 hours, followed by heating the resultant mixture to inactivate the remaining enzyme. The mixture thus obtained was in usual manner decolored with an activated charcoal, desalted with an ion-exchange resin, and concentrated to give a concentration of about 60%, d.s.b. The saccharide solution thus obtained contained 30.1% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)" a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 97% **trehalose,** d.s.b., and it was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with a seed crystal and gradually cooled to obtain a massecuite with a degree of crystallization of about 45%. The massecuite was sprayed from a nozzle equipped on the top of a spraying tower, at a pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air from the top of the spraying tower, and the resultant crystalline powder was collected on a metal wire netting conveyer provided on the basement of the spraying tower, gradually conveyed out from the tower while a stream of 40.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an aging tower and aged for 10 hours while sending hot air thereto to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline **trehalose**. The product exhibits no substantial hygroscopicity and has a satisfiable handleability, and these properties render it arbitrarily useful in food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
A seed culture of **Sulfolobus** solfataricus (ATCC 35091) was inoculated in a nutrient culture medium and incubated by a fermenter for about 42 hours in accordance with the method in Experiment 3. After completion of the incubation, the resultant culture was concentrated with an SF-membrane and centrifuged to recover cells, which were then disrupted with ultrasonic to

obtain an supernatant. The resultant supernatant was salted out with ammonium sulfate, dialyzed, and subjected to an ion-exchange column and hydrophobic column chromatography to obtain an enzyme solution containing 19.0 units/ml of a partially purified enzyme preparation having a specific activity of about 18 units/mg protein. In accordance with the method in Example A-3, the suspension of potato starch having a concentration of 30% was treated with "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, a maltotetraose forming amylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and ".alpha.-amylase 2A", .alpha.-amylase commercialized by Ueda Chemical Co., Tokyo, Japan, and autoclaved at 120.degree. C., cooled to 65.degree. C., admixed with 2 units/g starch of the above thermostable non-reducing saccharide-forming enzyme, and subjected to an enzymatic reaction for 64 hours. The resultant mixture was kept at 97.degree. C. for 30 min to inactivate the remaining enzyme. In accordance with the method in Example A-5, the resultant solution was subjected to the action of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., decolored, desalted and concentrated into an about 60% solution. The saccharide solution thus obtained contained about 23% **trehalose,** d.s.b. In accordance with the method in Example A-5, the saccharide solution was fractionated on column chromatography using a strongly-acidic action-exchange resin to obtain fractions rich in **trehalose**. The fractions containing about 95% **trehalose,** d.s.b., were pooled, placed in a vessel and boiled down under a reduced pressure into a syrup with a moisture content of about 4.0%. The syrup was placed in a crystallizer and admixed with one % of anhydrous crystalline **trehalose,** as a seed crystal, with respect to the syrup, d.s.b., followed by crystallizing the syrup at 95.degree. C. for 5 min while stirring. The resultant was transferred to an aluminum container and aged at 100.degree.. C. for 6 hours to form a block. The resultant block was pulverized by a cutting machine and subjected to a fluidized-bed drying to obtain a powdery anhydrous crystalline **trehalose** with a moisture content of about 0.3 w/w %. The product can be arbitrarily used in hydrous matters such as food products, cosmetics and pharmaceuticals, and their material and intermediates as a desiccant, as well as a white powdery sweetener with a high-quality and mild sweetness.

DEPR:
Three parts by weight of gum base was melted by heating until it softened, and the resultant was admixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method in Example A-5, and further admixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was kneaded by a roll in usual manner, formed and packed to obtain the desired product. The product is a chewing gum having a satisfiable texture and taste.

DEPR:
One part by weight of 40% "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was admixed with 2 parts by weight of a powder containing hydrous crystalline **trehalose** prepared by the method in Example A-5, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfiable taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:
Egg yolks prepared from fresh eggs were sterilized at 60.degree.-64.degree. C. by a plate heater, and the resultant liquid was admixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method in Example A-6 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was allowing to convert into hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk. The product can be arbitrarily used as a material for confectioneries for premixes, sherbets, ice cream and emulsifiers, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings. The product can be also used as a skin refiner and hair restorer.

DEPR:
To a column of an immobilized anti-human interferon-.alpha. antibody was fed in usual manner a natural human interferon-.alpha. preparation, commercialized by Hayashibara Biochemical Laboratories Inc., Okayama, Japan to adsorb the interferon-.alpha., and fed with a buffer containing calf serum albumin as a stabilizer, followed by removing an excessive amount of the albumin. Thereafter the interferon-.alpha. was eluted with a physiological saline containing 5% of a powdery hydrous crystalline **trehalose,** d.s.b., obtained by the method in Example A-5, while varying the pH of the physiological saline. The resultant eluate was filtered by a membrane, and the filtrate was dehydrated by about 20-fold volumes of "FINETOSE.RTM.", an anhydrous crystalline maltose powder commercialized by Hayashibara Shoji Inc., Okayama, Japan, followed by pulverizing the resultant dehydrated product and tabletting the resultant by a tabletting machine into tablets containing about 150 units of the natural human interferon-.alpha. per one tablet, 200 mg weight. The product can be orally administered as a sublingual tablet to patients at a dose of 1-10 tablets/adult/day, and arbitrarily used to treat viral diseases, allergys, rheumatisms, diabetes and malignant tumors. More particularly, the product can be suitably used as a therapeutic agent for AIDS and hepatitis, the number of patients of which has been remarkably increased. The **trehalose** and anhydrous crystalline maltose incorporated in the product act as a stabilizer, so that the natural human interferon-.alpha. is well retained its activity for a relatively-long period of time even at an ambient temperature.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method in Example A-5, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose,** one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to obtain a sugar coated tablet having a satisfiable gloss and appearance. The product has a relatively-high shock tolerance and retains its high quality for a relatively-long period of time.

DEPR:
A composition consisting of 500 parts by weight of a powder hydrous crystalline **trehalose** obtained by the method in Example A-5, 270 parts by weight of dried yolk, 209 parts by weight of defatted milk, 4.4 parts by weight of sodium chloride, 1.8 parts by weight of potassium chloride, 4 parts by weight of magnesium sulfate, 0.01 part by weight of thiamine, 0.1 part by weight of sodium ascorbate, 0.6 parts by weight of vitamin E acetate and 0.04 parts by weight of nicotine amide was prepared, and the composition was divided into 25 g aliquot in small moistureproof laminated aluminum packs which were then heat-sealed. One pack of the product is dissolved in about 150-300 ml water and the resultant solution is usable as an a liquid supplemental nutrition parenterally administrable to the nasal cavity, stomach or intestine.

DEPR:
Two hundred parts by weight of powder hydrous crystalline **trehalose** obtained by the method in Example A-5 and 300 parts by weight of crystalline maltose were admixed with 50 parts by weight of methanol containing 3 parts by weight of iodine, and the resultant was mixed with 200 parts by weight of 10 w/v % pullulan to obtain a traumatic ointment which has an appropriate extensity and adhesiveness. The product shortens a therapeutic period and cure traumas without a scar by reason that the iodine incorporated in the product exhibits sterilizing effects and also the **trehalose** incorporated in the product supplements nutrition into traumas.

DEPR:
As evident from above, the present novel thermostable non-reducing saccharide readily realizes an enzymatic reaction at the temperature of over 55.degree. C. wherein partial starch hydrolysates are converted at a satisfactorily-high yield into non-reducing saccharides having the same degree of glucose polymerization as that of the partial starch hydrolysates without a fear of contamination. The non-reducing saccharides, which can be readily separated

and purified, and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, have a satisfiable stability, quality and mild sweetness. These non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be arbitrarily used in compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thus, the present invention provides a novel technique to prepare in an industrial-scale and at a relatively-low cost non-reducing saccharides having a **trehalose** structure as an end unit, which could not have been readily obtained in spite of their great demands, by using reducing partial starch hydrolysates prepared from starch as a cheap and abundant source, as well as to prepare relatively-low reducing saccharides containing the non-reducing saccharides, and **trehalose** prepared from these saccharides. The present invention has a great influence on the fields such as food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomable.

DEPC:
Preparation of thermostable non-reducing saccharide-forming enzyme from **Sulfolobus acidocaldarius** ATCC 33909

DEPC:
Preparation of thermostable non-reducing saccharide-forming enzyme from other microorganisms of the genus **Sulfolobus**

DEPV:
Forming a non-reducing saccharide having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

DETL:
TABLE 1 ______________________________________ Saccharides in Saccharide Substrate reaction mixture composition (%) ______________________________________ Glucose Glucose 100.0 Maltose Maltose 100.0 Maltotriose Glucose 9.2 Maltose 18.4 Maltotriose 42.2 .alpha.-glucosyl **trehalose** 30.2 Maltotetraose Glucose 6.7 Maltose 2.7 Maltotriose 9.0 Maltotetraose 16.2 .alpha.-glucosyl **trehalose** 8.2 .alpha.-maltosyl **trehalose** 57.2 Maltopentaose Glucose 0.7 Maltotetraose 2.0 Maltopentaose 22.9 .alpha.-maltosyl **trehalose** 0.9 .alpha.-maltotriosyl **trehalose** 73.5 Maltohexaose Glucose 0.9 Maltopentaose 2.2 Maltohexaose 23.1 .alpha.-maltotriosyl **trehalose** 5.6 .alpha.-maltotetraosyl **trehalose** 68.2 Maltoheptaose Glucose 1.0 Maltohexaose 1.4 Maltoheptaose 23.4 .alpha.-maltotetraosyl **trehalose** 4.2 .alpha.-maltopentaosyl **trehalose** 70.0 ______________________________________

DETL:
TABLE 2

____

Enzyme activity in eluate from Optimum Thermal ion-exchange temperature Optimum stability pH Microorganism column (unit) (.degree.C.) pH (.degree.C.) stability

____

Sulfolobus 440 About 75.degree. C. About 5.0-5.5 Up to About 4.5-9.5 **acidocaldarius** about 85.degree. C. (ATCC 33909) **Sulfolobus** 370 About 75.degree. C. About 5.0-5.5 Up to About 4.5-9.5 **acidocaldarius** about 85.degree. C. (ATCC 49426) **Sulfolobus** 210 About 75.degree. C. About 5.0-5.5 Up to About 4.0-8.5 solfataricus about 85.degree. C. (ATCC 35091) **Sulfolobus** 95 About 75.degree. C. About 5.0-5.5 Up to About 4.0-8.5 solfataricus about 85.degree. C. (ATCC 35092)

____

CLPR:
1. A purified enzyme which forms, without requiring **trehalose** as a substrate, a non-reducing saccharide having a **trehalose** structure as an end unit and

consisting of **trehalose** and glucose units when allowed to act on a reducing partial starch hydrolysate, wherein the purified enzyme is not inactivated at a temperature in the range of 55.degree.-70.degree. C.

CLPR:
5. The purified enzyme of claim 4, wherein said microorganism is a member selected from the group consisting of those of the genus **Sulfolobus** and mutants thereof.

CLPR:
8. The process of claim 7, wherein said microorganism is a member selected from the group consisting of those of the genus **Sulfolobus** and mutants thereof.

CLPR:
9. A process for producing a non-reducing saccharide having a **trehalose** structure as an end unit and consisting of **trehalose** and glucose units which comprises:

CLPR:
17. A process for preparing **trehalose** which comprises:

CLPR:
19. The process of claim 17, wherein said **trehalose** is a member selected from the group consisting of hydrous crystalline **trehalose,** anhydrous crystalline **trehalose,** and mixtures thereof.

CLPR:
20. The process of claim 17, wherein the resultant solution in step (c) is further subjected to column chromatography using a strongly-acidic cation-exchange resin to increase the content of **trehalose**.

CLPR:
21. A process for preparing a food product which comprises preparing **trehalose** according to the process of claim 17 and then incorporating the **trehalose** into a food material.

CLPR:
22. A process for preparing a cosmetic which comprises preparing **trehalose** according to the process of claim 17 and then incorporating the **trehalose** into a cosmetically-acceptable carrier.

CLPR:
23. A process for preparing a pharmaceutical which comprises preparing **trehalose** according to the process of claim 17 and then incorporating the **trehalose** into a pharmaceutically-acceptable carrier.

CLPV:
Without requiring **trehalose** as a substrate, forming a non-reducing saccharide, having a **trehalose** structure as an end unit and consisting of **trehalose** and glucose units, when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of at least 3;

CLPV:
(a) allowing the enzyme of claim 1 to act on a solution containing a reducing partial starch hydrolysate as a substrate to form a non-reducing saccharide having a **trehalose** structure as an end unit and consisting of **trehalose** and glucose units;

CLPV:
(b) allowing glucoamylase or .alpha.-glucosidase to act on the formed non-reducing saccharide to form **trehalose**; and

CLPV:
(c) recovering the resultant solution containing **trehalose** together with intact reducing partial starch hydrolysate.

ORPL:
L. Lama et al, "Thermostable amylolytic activity from **Sulfolobus** solfataricus",

Biotec Forum Europe, vol. 8, No. 1, 2 Feb. 1991, pp. 201-203.

USPT

US-CL-CURRENT: 536/123.1

US-PAT-NO: 5681826
DOCUMENT-IDENTIFIER: US 5681826 A
TITLE: Saccharide composition with reduced reducibility, and preparation and uses thereof
DATE-ISSUED: October 28, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/54,536/123.1
ABSTRACT:

A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction.

19 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

ABPL:
A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction..

BSPR:
The present invention relates to a saccharide composition with a reduced reducibility, and preparation and uses thereof, more particularly, to a saccharide composition with a reduced reducibility which comprises a sugar alcohol and a non-reducing saccharide consisting of **trehalose** and/or a saccharide having a **trehalose** structure.

BSPR:
Trehalose or .alpha.,.alpha.**-trehalose,** a non-reducing saccharide consisting of two glucopyranoside residues, has been well known. As is described in Advances in Carbohydrate Chemistry, Vol.18, pp.201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol.56, pp.3,213-3,215 (1990), **trehalose** is widely distributed in microorganisms, mushrooms, insects, etc., though the content is relatively low. Since non-reducing saccharides including **trehalose** do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, such non-reducing saccharides have been expected to be used without fear of causing unsatisfiable browning and deterioration, and the preparation of which has been in great demand.

BSPR:
Examples of conventional preparations of **trehalose** include a method as disclosed in Japanese Patent Laid-Open No.154,485/75 wherein microorganisms are utilized, and a conversion method as proposed in Japanese Patent Laid-Open No.216,695/83 wherein maltose is converted into **trehalose** by the combination use of maltose- and **trehalose**-phosphorylases. The former is, however, not suitable for the industrial-scale preparation of **trehalose** because the content of **trehalose** present in microorganisms as a starting material is usually lower

than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the present specification, unless specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, the concentration of maltose as a substrate could not be set to a relatively-high concentration; (ii) Since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) It is substantially difficult to retain the reaction systems stably and to continue their enzymatic reactions smoothly. Therefore, they have not been established as an industrial-scale preparation.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No.88, pp.67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been recognized to be scientifically almost impossible." so that an enzymatic preparation of **trehalose** from starch as a material has been recognized to be scientifically difficult.

BSPR:
To overcome such conventional drawbacks, the present inventors had disclosed in Japanese Patent Application No.349,216/93 a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure as an end unit from one or more reducing partial starch hydrolysates with a glucose polymerization degree of 3 or more (throughout the present specification, the enzyme is designated as "non-reducing saccharide-forming enzyme"). By using the non-reducing saccharide-forming enzyme, they established the preparation of non-reducing saccharides, which have a glucose polymerization degree of 3 or more and a **trehalose** structure as an end unit, from reducing partial starch hydrolysates, as well as establishing saccharide compositions with a reduced reducibility and the preparation of **trehalose** from these non-reducing saccharides and saccharide compositions.

BSPR:
The present inventors also disclosed in Japanese Patent Application No.79,291/94 a novel **trehalose**-releasing enzyme which specifically hydrolyses the linkage between a **trehalose** moiety and others in non-reducing saccharides having a glucose polymerization degree of 3 or more and a **trehalose** structure as an end unit (throughout the present specification, the enzyme is designated as "**trehalose**-releasing enzyme"), and established the preparation of **trehalose** in a relatively-high yield from reducing partial starch hydrolysates by the combination use of the aforesaid non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme. It was revealed that in the preparations of saccharide compositions having a **trehalose** structure and a glucose polymerization degree of 3 or more and those of non-reducing saccharides such as **trehalose,** intact reducing partial starch hydrolysates still remain in the final products, and reducing amylaceous saccharides such as glucose and maltose are newly formed. Required is to more reduce the reducibility of these saccharide compositions with a reduced reducibility which contain the aforesaid non-reducing saccharides and reducing saccharides.

BSPR:
The present invention is to provide saccharides having a **trehalose** structure or non-reducing saccharides having a **trehalose** structure as an end unit (hereinafter designated as ".alpha.-glycosyltrehalose" in the present specification), non-reducing saccharides wherein one or more glucose residues bind to the two glucopyranoside residues of **trehalose** (hereinafter designated as ".alpha.-glycosyl .alpha.-glycoside" in the present specification), and saccharides prepared by more reducing the reducibility of those with a relatively-low reducibility which contain reducing saccharides and non-reducing saccharides such as **trehalose**. The present invention is to also provide the preparation and uses of these saccharides.

BSPR:
As a result, they found that reducing amylaceous saccharides can be readily converted into their corresponding sugar alcohols by hydrogenating saccharide

compositions with a reduced reducibility comprising the reducing amylaceous saccharides and other non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure without fear of the affection of the non-reducing saccharides, and that the reducibility of the saccharide compositions as a material is lowered or even substantially diminished.

BSPR:
The present inventors studied on the preparation of the materials usable in the present invention, i.e. saccharide compositions with a relatively-low reducibility which comprise reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, and have found that compositions which are obtainable by allowing a non-reducing saccharide-forming enzyme together with or without a **trehalose**-releasing enzyme to act on reducing partial starch hydrolysates with a glucose polymerization degree of 3 or more can be arbitrarily used. More particularly, saccharide compositions with a low reducibility, which are obtainable by allowing starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on liquefied starch solutions when a non-reducing saccharide-forming enzyme is allowed to act on the solutions together with or without a **trehalose**-releasing enzyme, are satisfactorily used. Furthermore, the present inventors found that saccharide compositions with a low reducibility, which are obtainable by allowing a maltose-**trehalose** converting enzyme to act on amylaceous substances, as disclosed in Japanese Patent Application No.144,092/94, titled "Maltose-**trehalose** converting enzyme, and preparation and uses thereof", applied for by the present applicant on the day of Jun. 3, 1994, can be arbitrarily used in the present invention. Thus, they accomplished this invention and revealed that, in the process for preparing such saccharide compositions with a reduced reducibility wherein a non-reducing saccharide-forming enzyme is allowed to act on amylaceous solutions with a DE less than 15, saccharide compositions with a low reducibility, which contain non-reducing saccharides obtainable by allowing a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on the amylaceous solutions, can be suitably used as a material saccharide in the present invention because they have an extremely reduced molecular weight and viscosity and a satisfactory handleability without substantial increment of their initial reducibility than those prepared by the sole use of the non-reducing saccharide-forming enzyme. They also found that the **trehalose** content in the saccharide compositions with a relatively-low reducibility increases when glucoamylase acts on them. In addition, they found that, in the process for preparing **trehalose** by allowing a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme to act on liquefied starch solutions with a relatively-low DE, preferably, those with a DE less than 15, **trehalose** which is obtainable by using a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase can be suitably used as a material in the present invention because such **trehalose** is obtained in a relatively-high yield as compared with that obtained by the sole use of the non-reducing saccharide-forming enzyme. Furthermore, it was found that saccharide compositions of **trehalose** and maltose, which are obtainable by allowing a maltose-**trehalose** converting enzyme to act on maltose, can be arbitrarily used in the present invention. The resultant saccharide compositions with a low reducibility and rich in non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure can be readily hydrogenated. These saccharide compositions are substantially free of reducibility or they have a dextrose equivalent (DE) value less than 1, and have a satisfactory stability, handleability and wide applicability. Thus, they can be arbitrarily used in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DRPR:
FIG. 9 shows the elution patterns of a **trehalose**-releasing enzyme and a non-reducing saccharide-forming enzyme usable in the present invention on column chromatography using "TOYOPEARL".

DRPR:
FIG. 10 shows the influence of temperature on a **trehalose**-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 12 shows the influence of temperature on the stability of a

trehalose-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 13 shows the influence of pH on the stability of a **trehalose**-releasing enzyme derived from Rhizobium sp. M-11.

DRPR:
FIG. 14 shows the influence of temperature on the activity of a **trehalose**-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 15 shows the influence of pH on the activity of a **trehalose**-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 16 shows the influence of temperature on the activity of a **trehalose**-releasing enzyme derived from Arthrobacter sp. Q36.

DRPR:
FIG. 17 shows the influence of pH on the activity of a **trehalose**-releasing enzyme derived from Arthrobacter sp. Q36.

DEPR:
The non-reducing saccharide-forming enzymes usable in the present invention include those which can form .alpha.-glycosyltrehalose from one or more reducing amylaceous partial starch hydrolysates selected from those with a glucose polymerization degree of 3 or more which are contained in liquefied starch solutions with a relatively-low DE. Examples of such enzymes are those derived from microorganisms of the genera Rhizobium, Arthrobacter, Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium, Mycobacterium and Terrabacter as disclosed in Japanese Patent Application No.349,216/93. If necessary, thermostable non-reducing saccharide-forming enzymes can be arbitrarily used in the present invention. For example, a thermostable non-reducing saccharide-forming enzyme derived from a microorganism of the genus **Sulfolobus** as disclosed in Japanese Patent Application No.166,011/94, titled "Thermostable non-reducing saccharide-forming enzyme, and its preparation and uses", applied for by the present applicant on the day of Jun. 24, 1994, can be arbitrarily used. Any enzyme, which specifically hydrolyzes the linkage between a **trehalose** moiety and others in .alpha.-glycosyltrehalose formed by allowing a non-reducing saccharide-forming enzyme to act on a liquefied starch solution, can be used as a **trehalose**-releasing enzyme in the present invention: For example, those derived from microorganisms of the genera Rhizobium, Arthrobacter, Brevibacterium and Micrococcus as disclosed in Japanese Patent Application No.79,291/94 can be arbitrarily used. If necessary, thermostable **trehalose**-releasing enzymes such as that derived from a microorganism of the genus **Sulfolobus** as disclosed in Japanese Patent Application No.166,126/94, applied for by the present applicant on the day of Jun. 25, 1994, can be arbitrarily used in the present invention.

DEPR:
Any enzyme can be arbitrarily used in the present invention as a maltose-**trehalose** converting enzyme as long as it forms trehalose: Examples of such an enzyme are those derived from microorganisms of the genera Pimerobacter, Pseudomonas and Thermus as disclosed in Japanese Patent Application No.144,092/94, titled "Maltose-**trehalose** converting enzyme, and preparation and uses thereof", applied for by the present applicant on the day of Jun. 3, 1994. The methods used for preparing the non-reducing saccharide-forming enzymes, **trehalose**-releasing enzymes, and maltose-**trehalose** converting enzymes in the present invention are those which comprise culturing microorganisms capable of forming such enzymes in nutrient culture media, and collecting the formed enzymes.

DEPR:
The **trehalose**-releasing enzymes usable in the present invention generally have the following physicochemical properties:.

DEPR:
The activity of the **trehalose**-releasing enzymes is assayed as follows: One ml

of an enzyme solution is added to 4 ml of 1.25 w/v % maltotriosyl **trehalose** alias .alpha.-maltotetraosyl .alpha.-D-glucoside in 50 mM phosphate buffer (pH 7.0), and the mixture solution is incubated at 40.degree. C. for 30 min. To the resultant reaction mixture is added a copper solution for the Somogyi reaction to suspend the enzymatic reaction, followed by the determination of the reducing power on the Somogyi-Nelson's method. As a control, an enzyme solution, which was preheated at 100.degree. C. for 10 min to inactivate the enzyme, is assayed similarly as above. One unit activity of the enzyme is defined as the amount of enzyme which increases the reducing power of that of one .mu.mole of glucose per minute when assayed with the above-mentioned assay.

DEPR:
The maltose-**trehalose** releasing enzymes usable in the present invention have the following physicochemical properties:

DEPR:
The activity of the maltose-**trehalose** converting enzymes is assayed as follows: One ml of an enzyme solution is added to one ml of 20 w/v % maltose as a substrate in 10 mM phosphate buffer (pH 7.0), and the mixture solution is incubated at 25.degree. C. for 60 min, followed by heating the solution at 100.degree. C. for 10 min to suspend the enzymatic reaction. To the resultant reaction mixture is precisely diluted by 11-fold with 50 mM phosphate buffer (pH 7.5), and 0.4 ml of the diluted solution is admixed with 0.1 ml of an enzyme solution containing one unit/ml **trehalose**. The resultant solution is incubated at 45.degree. C. for 120 min, followed by determining the amount of glucose by the glucose-oxidase method. As a control, by using **trehalose** and an enzyme solution, which were preheated at 100.degree. C. for 10 min to inactivate the enzymes, the resultant enzyme solution is assayed similarly as above. With the above assay, the content of **trehalose**, formed by the maltose-**trehalose** converting enzyme, is determined based on the amount of the formed glucose, and one unit activity of the enzyme is defined as the amount of enzyme which forms one .mu.mole of **trehalose** per minute.

DEPR:
The liquefied starch solutions thus obtained can be subjected to the action of a non-reducing saccharide-forming enzyme together with a starch debranching enzyme and/or a cyclomaltodextrin glucanotransferase, or to the action of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme together with a starch debranching enzyme and/or a cyclomaltodextrin glucanotransferase under the pH- and temperature-conditions which allow these enzymatic reactions to proceed, for example, a pH of 4-10, preferably, of 5-8, and a temperature of about 10.degree.-80.degree. C., preferably, of about 30.degree.-70.degree. C. The order of the enzymes to be added to the liquefied starch solutions is not specifically restricted to, for example, one or more of these enzymes can be first added to the solutions, then the remaining enzyme(s) is added to, or all the enzymes can be added to at the same time.

DEPR:
The amounts of the enzymes to be added can be chosen depending upon the enzymatic conditions and reaction times. Usually, they are chosen from (i) about 0.01-100 units/g substrate, d.s.b., in liquefied starch solutions for the enzymatic reaction of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme, (ii) about 1-10,000 units/g substrate, d.s.b., for starch debranching enzymes, and (iii) about 0.05-500 units/g substrate, d.s.b., for cyclomaltodextrin glucanotransferases. The resultant saccharide compositions with a reduced reducibility, which comprise non-reducing saccharides and reducing amylaceous saccharides, are prepared by subjecting liquefied starch solutions to the action of starch debranching enzymes and/or cyclomaltodextrin glucanotransferases and non-reducing saccharide-forming enzymes together with or without **trehalose**-releasing enzymes so that the saccharide compositions have characters that they contain **trehalose** in quantity or considerably-large amounts of relatively-low molecular weight .alpha.-glycosyltrehalose and/or .alpha.-glycosyl .alpha.-glycoside, and that they can be arbitrarily used as a material saccharide composition with a reduced reducibility in the present invention. The .alpha.-glycosyl .alpha.-glycoside is a name given to a compound such as .alpha.-D-oligoglycosyl .alpha.-D-oligoglucoside as disclosed in Japanese Patent Application No.54,377/94 applied for by the present applicant.

DEPR:
If necessary, the resultant saccharide compositions with a reduced reducibility, which contain non-reducing saccharides having a **trehalose** structure, can be further processed into the material saccharide composition with a reduced reducibility used in the present invention by hydrolyzing them with amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase or .alpha.-glucosidase to control their sweetness and/or lower their viscosity.

DEPR:
The saccharide compositions thus obtained can be arbitrarily used as a material saccharide composition in the present invention. For example, those rich in non-reducing saccharides consisting of **trehalose** and saccharides having a **trehalose** structure, i.e. those which contain 20% or more, preferably, 40% or more, more preferably, 60% or more of **trehalose,** d.s.b., and have a relatively-low DE, usually, a DE less than 70, preferably, a DE less than 50, more preferably, a DE less than 30, are satisfactorily used. Varying dependently on their compositions, the material saccharide compositions used in the present invention have features that they have a relatively-low DE regardless of their large amount of non-reducing saccharides, relatively-low molecular weight substances to be tasted, and relatively-low viscosity, and that they facilitate the hydrogenation and the subsequent processes such as purification and concentration wherein the amount of hydrogen which is required for the hydrogenation is reduced by a large margin.

DEPR:
Any method for hydrogenating the resultant saccharide compositions with a reduced reducibility, which comprise reducing saccharides and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, can be used in the present invention as long as it does not decompose the non-reducing saccharides but hydrogenates saccharides into sugar alcohols. For example, the material saccharide compositions are prepared into 30-70% aqueous solutions, transferred to an autoclave, mixed with about 8-10% Raney nickel as a catalyst, and heated up to a temperature of 90.degree.-150.degree. C. under stirring conditions to terminate the hydrogenation or, preferably, to partially hydrogenate the contents until they show a DE less than 0.5, followed by removing the Raney nickel. The resultant mixtures were decolored with an activated charcoal in usual manner, desalted with an ion-exchange resin, and concentrated into syrupy products. If necessary, the syrupy products are arbitrarily dried into powdery products, or crystallized into crystalline powders containing **trehalose** crystal. The resultant saccharide compositions with a reduced reducibility contain non-reducing saccharides, which consist of **trehalose** and/or non-reducing saccharides having a **trehalose** structure, such as .alpha.-glycosyltrehalose and .alpha.-glycosyl .alpha.-glycoside, and one or more sugar alcohols such as sorbitol, maltitol, maltotriitol, maltotetraiol and maltopentaitol.

DEPR:
The saccharide compositions according to the present inventions form relatively-low molecular weight non-reducing oligosaccharides and maltooligosaccharides when hydrolyzed with amylases such as .alpha.-amylase derived from pancreas. The oligosaccharides are readily hydrolyzed with .alpha.-glucosidase and enzymes in small intestines into glucose and **trehalose** which is then readily hydrolyzed with **trehalose** into glucose molecules. Thus, the present saccharide compositions are readily assimilated, absorbed and utilized by living bodies when orally administered. Furthermore, they are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
Fifty mg aliquots of non-reducing saccharide preparations P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for 6 hours. HPLC analysis only detected glucose and **trehalose** in all resultant mixtures. The percentages and the molecular ratios of glucose to **trehalose** in each saccharide are in Table 5.

DEPR:
As is evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one glucose molecule and one **trehalose** molecule; P II, hydrolyzed into 2 glucose molecules and one **trehalose** molecule; (iii) P III, hydrolyzed into 3 glucose molecules and one **trehalose** molecule; (iv) P IV, hydrolyzed into 4 glucose molecules and one **trehalose** molecule; and (v) P V, hydrolyzed into 5 glucose molecules and one **trehalose** molecule.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more glucose molecules bound to one **trehalose** molecule via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing saccharide which has a glucose polymerization degree of 3 (DP 3) and consists of one glucose molecule bound to one **trehalose** molecule; P II, a non-reducing saccharide which has DP 4 and consists of 2 glucose molecules bound to one **trehalose** molecule; P III, a non-reducing saccharide which has DP 5 and consists of 3 glucose molecules bound to one **trehalose** molecule; P IV, a non-reducing saccharide which has DP 6 and consists of 4 glucose molecules bound to one **trehalose** molecule; and P V, a non-reducing saccharide which has DP 7 and consists of 5 glucose molecules bound to one **trehalose** molecule. It was revealed that, when .beta.-amylase was act on these non-reducing saccharides similarly as in glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one maltose molecule and one P I molecule, one maltose molecule and one P II molecule, and 2 maltose molecules and one P I molecule.

DEPR:
Based on these results, it was concluded that the enzymatic reaction mechanism of the present non-reducing saccharide-forming enzymes is an intramolecular conversion reaction without changing the molecular weights of substrates, i.e. an intramolecular conversion reaction without changing the glucose polymerization degrees of substrates. It was also concluded that the non-reducing saccharides P I, P II, P III, P IV and P V were respectively .alpha.-glycosyltrehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.-**trehalose** residue) of .alpha.-glucosyltrehalose, .alpha.-maltosyltrehalose, .alpha.-maltotriosyltrehalose, .alpha.-maltotetraosyltrehalose and .alpha.-maltopentaosyltrehalose.

DEPR:
As is evident from the results in Tables 7 and 8, it was revealed that, similarly as in Experiment 6 wherein glucoamylase is used, the saccharide preparations P I, P II, P III, P IV and P V were hydrolyzed by the .alpha.-glucosidase and the rat intestinal acetone powder into glucose and **trehalose**.

DEPR:
To the resultant hydrolysate obtained by the .alpha.-glucosidase or the rat intestinal acetone powder was added one unit **trehalose** derived from pig kidney, a product of Sigma Chemical Company, St., Louis, USA, and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC revealing that **trehalose**, formed from the saccharide preparations P I, P II, P III, P IV and P V, was hydrolyzed by **trehalose** into glucose molecules.

DEPR:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is hydrolyzed by glucoamylase into one **trehalose** molecule and 2 glucose molecules.

DEPR:
Based on these results, it was concluded that the non-reducing saccharide-forming enzyme used in the present invention is an enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates into a non-reducing end unit, i.e. a **trehalose** residue or a

trehalose structure.

DEPR:
The followings are the explanations of **trehalose**-releasing enzymes from Rhizobium sp. M-11 and Arthrobacter sp. Q36, as well as from known microorganisms:

DEPR:
The activities of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme accumulated in the culture were respectively about 1.5 units/ml and about 2 units/ml. A portion of the culture was centrifuged into cells and a culture supernatant, and the cells were suspended in 50 mM phosphate buffer (pH 7.0) to give the same volume of the portion, followed by assaying the enzyme activities of the cell suspension and the culture supernatant. The activities of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme in the cell suspension were respectively about 0.6 units/ml and about 0.8 units/ml, and the culture supernatant contained about 0.9 units/ml of the non-reducing saccharide-forming enzyme and about 1.2 units/ml of the **trehalose**-releasing enzyme.

DEPR:
The objective non-reducing saccharide-forming enzyme and **trehalose**-releasing enzyme were adsorbed on the ion-exchanger, and eluted separately from the column with a fresh preparation of the same phosphate buffer supplemented with salt at different salt concentrations. The elution pattern from the column or the column chromatogram is in FIG. 9. The non-reducing saccharide-forming enzyme was eluted from the column at a salt concentration of about 0.2M, while the **trehalose**-releasing enzyme was eluted from the column at a salt concentration of about 0.3M. The fractions containing either of the objective enzymes were separately pooled and purified as follows:

DEPR:
Fractions with a **trehalose**-releasing enzyme activity eluted from the column of "DEAE-TOYOPEARL.RTM.", were pooled and treated similarly as in the purification steps used in the preparation of the non-reducing saccharide-forming enzyme in such a manner that they were dialyzed against a buffer containing 2M ammonium sulfate, and successively subjected to hydrophobic column chromatography and gel filtration chromatography.

DEPR:
The total enzyme activity, specific activity and yield of the non-reducing saccharide-forming enzyme in each purification step are in Table 11, while those of the **trehalose**-releasing enzyme are in Table 12.

DEPR:
A portion of a purified **trehalose**-releasing enzyme preparation, obtained by the method in Experiment 15, was electrophoresed in a 10% sodium dodecylsulfate polyacrylamide gel, and determined for molecular weight by comparing it with marker proteins commercialized by Japan Bio-Rad Laboratories, Tokyo, Japan, revealing that it has a molecular weight of about 58,000-68,000 daltons.

DEPR:
.alpha.-Glycosyltrehalose as a substrate was prepared in accordance with the method in Experiment 4: To a 20% aqueous solution of a reducing partial starch hydrolysate selected from maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was added 2 units/g substrate, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 15, subjected to an enzymatic reaction at 40.degree. C. and pH 7.0 for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, filtered, decolored, desalted and concentrated into a high saccharide content solution which was then column chromatographed by using "XT-1016 in Na.sup.+ -form, an ion-exchanger commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan. In the column chromatography, the ion-exchanger was packed in 3-jacketed stainless steel columns, having an inner diameter of 2.0 cm and a length of one m each, which were then cascaded in series, heated to give the inner column temperature of 55.degree. C., applied with 5 v/v % of the concentrated saccharide solution to the resin while keeping at 55.degree. C., and fed with 55.degree. C. hot water at SV 0.13 to obtain

high-purity non-reducing saccharides having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more. Among the resultant high-purity preparations, the purities of the preparations of glucosyltrehalose, maltotriosyl-**trehalose,** maltotriosyltrehalose, maltotetraosyltrehalose and maltopentaosyltrehalose were respectively 97.6%, 98.6%, 99.6%, 98.3% and 98.1%, d.s.b.

DEPR:
An aqueous solution containing 20%, d.s.b., of one of the above 5 non-reducing saccharide preparations, namely .alpha.-glycosyltrehalose preparations, was prepared, followed by mixing it with 2 units/g substrate, d.s.b., of the purified **trehalose**-releasing enzyme obtained in Experiment 15, and subjecting the resultant to an enzymatic reaction at 40.degree. C. and pH 7.0 for 48 hours. The resultant each reaction mixture was desalted, and analyzed for saccharide composition on HPLC using "WAKOBEADS WB-T-330 column", a column of Wako Pure Chemical Industries Ltd., Tokyo, Japan. As a control, a fresh preparation of the same **trehalose**-releasing enzyme was allowed to act on maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose, and the resultant each reaction mixture was analyzed for saccharide composition on HPLC. The results are in Table 13.

DEPR:
These results confirm that the **trehalose**-releasing enzyme usable in the present invention is an enzyme which has a new reaction mechanism of specifically hydrolyzing the linkage between a **trehalose** moiety and other glycosyl moiety in .alpha.-glycosyltrehalose to release **trehalose**.

DEPR:
To purify **trehalose** in each reaction mixture, it was subjected to column chromatography using a column packed with "XT-1016", a strong-acid cation exchange resin in Na.sup.+ -form commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, followed by recovering fractions containing 97% or more of **trehalose**. The fractions were pooled and concentrated into an about 65% solution which was then allowed to stand at 25.degree. C. for 2 days to crystallize **trehalose** into hydrous crystalline **trehalose,** followed by separating and drying it in vacuo to obtain a high-purity **trehalose** preparation with a purity of 99% or more, d.s.b. The yields of **trehalose** from glucosyltrehalose, maltosyltrehalose, maltotriosyltrehalose, maltotetraosyltrehalose and maltopentaosyltrehalose used as a substrate were respectively 9.5%, 14.9%, 16.0%, 18.5% and 17.7%, d.s.b. The high-purity **trehalose** preparations and a commercially available **trehalose** specimen as a standard were studied for melting point, heat of fusion, specific rotation, infrared absorption spectrum, powdery X-ray diffraction pattern, and readiness of hydrolysis by a **trehalose** specimen derived from pig kidney, commercialized by Sigma Chemical Co., St. Louise, USA. As a result, every **trehalose** preparation showed a melting point of 97.0.degree..+-.0.5.degree. C., a heat of fusion of 57.8.+-.1.2 kJ/mole and a specific rotation of +182.degree..+-.1.1.degree., and these values well corresponded with those of the standard **trehalose** specimen, and the infrared absorption spectra and powdery X-ray diffraction patterns of the **trehalose** preparations also well corresponded with those of the standard **trehalose** specimen. Similarly as the standard **trehalose** specimen, the **trehalose** preparations were decomposed into glucose molecules. As is evident from these results, it was identified that the saccharide, which is formed by allowing the **trehalose**-releasing enzyme to act on .alpha.-glycosyltrehalose, is **trehalose**.

DEPR:
Either of the resultant reducing partial starch hydrolysates or maltotriose having a glucose polymerization degree of 3, as a substrate, was dissolved in 10 mM phosphate buffer (pH 7.0) into a one % solution which was then mixed with a purified non-reducing saccharide-forming enzyme and a purified **trehalose**-releasing enzyme, which were prepared by the method in Experiment 15, in respective amounts of 4 units/g substrate, d.s.b., and subjected to an enzymatic reaction at 40.degree. C. for 24 hours. After completion of the reaction, a portion of the resultant reaction mixture was desalted and analyzed on HPLC.

DEPR:

As is shown in Table 14, in the case of using as a substrate maltotriose having a glucose polymerization degree of 3, the **trehalose** yield after the enzymatic reactions of the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme was as low as 4.2%, while in the case of using as a substrate partial starch hydrolysates having a glucose polymerization degree of 10-34.1, the **trehalose** yield was high, i.e. 66.1-80.8%. It was found that the higher the glucose polymerization degree of the reducing partial starch hydrolysates as a substrate, the higher the purity of **trehalose** in the resultant reaction mixtures. It was also found that the purity of **trehalose** in the resultant reaction mixture can be more increased by allowing glucoamylase to act on the reaction mixtures, which were prepared by these enzymes, to hydrolyze the remaining non-reducing saccharides, having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more, into **trehalose** and glucose molecules.

DEPR:
A solution, containing one % of glycine, 10% of a high-purity **trehalose** preparation with a purity of 99.5%, d.s.b., obtained by the method in Experiment 17, and 50 mM phosphate buffer (pH 7.0), was kept at 100.degree. C. for 90 min, followed by cooling the resultant solution and determining the absorbance at a wave length of 480 nm in a 1-cm cell. As a control, glucose and maltose were similarly treated as above, and the resultants were determined for absorbance at a wave length of 480 nm. The results are in Table 15.

DEPR:
As is evident from the results in Table 15, it was revealed that the **trehalose** preparation was slightly colored on the maillard reaction, i.e. the coloration degree was only about 0.4-0.6% of that of glucose or maltose. The results show that the present **trehalose** preparation is substantially free from the maillard reaction. Thus, the preparation is a saccharide which does not substantially deteriorate amino acids even when mixed with them.

DEPR:
In accordance with the method as reported by H. Atsuji et al. in "Rinsho-Eiyo", Vol.41, No.2, pp.200-208 (1972), 30 g of a high-purity **trehalose** preparation with a purity of 99.5%, d.s.b., obtained by the method in Experiment 17 was dissolved in water into a 20 w/v % aqueous solution which was then orally administered to 6 healthy male volunteers, 26-, 27-, 28-, 29-, 30- and 31-year-old. The volunteers were collected their blood at a prescribed time interval, and each collected blood was assayed for blood sugar- and insulin-levels. As a control Glucose was used. As a result, the **trehalose** preparation showed the same dynamics as glucose, i.e. the blood sugar- and insulin-levels showed their maxima at an about 0.5-1 hour after their administrations. It was revealed that the **trehalose** preparation is readily assimilated, absorbed, metabolized and utilized by the body as an energy source.

DEPR:
Similarly as in Experiment 14, a seed culture of Arthrobacter sp. Q36 (FERM BP-4316) was cultured by a fermenter for about 72 hours in place of Rhizobium sp. M-11 (FERM BP-4130). The activities of a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme in the resultant culture were respectively about 1.3 units/ml and about 1.8 units/ml. Similarly as in Experiment 14, a cell suspension and a culture supernatant, prepared from the resultant culture, were assayed revealing that the former had about 0.5 units/ml of the non-reducing saccharide-forming enzyme and about 0.5 units/ml of the **trehalose**-releasing enzyme, and that the latter had about 0.8 units/ml of the non-reducing saccharide-forming enzyme and about 1.3 units/ml of the **trehalose**-releasing enzyme.

DEPR:
By using an about 18 L of a culture containing enzymes obtained by the method in Experiment 22, purified enzyme preparations were obtained similarly as the method in Experiment 15. The results in each purification step for a non-reducing saccharide-forming enzyme and a **trehalose**-releasing enzyme are respectively in Tables 16 and 17.

DEPR:

Purified enzyme preparations of the non-reducing saccharide-forming enzyme and the trehalose-releasing enzyme, obtained as the eluates from gel filtration columns in Tables 16 and 17, were determined for purity on electrophoresis similarly as in Experiment 15. As a result, they were respectively found as a single band revealing that they were electrophoretically homogenous and relatively-high in purity.

DEPR:
A purified trehalose-releasing enzyme preparation, obtained by the method in Experiment 23, was determined for molecular weight on SDS-PAGE to give about 57,000-67,000 daltons. The pI of the enzyme preparation was determined on isoelectrophoresis similarly as in Experiment 3 revealing that it is about 3.6-4.6. The influences of temperature and pH on the enzyme activity, as well as the thermal stability and pH stability, were studied similarly as in Experiment 16. The results of them are respectively in FIGS. 14-17.

DEPR:
By using a purified enzyme preparation obtained by the method in Experiment 23, trehalose was prepared from non-reducing saccharides having a trehalose structure as an end unit and a glucose polymerization degree of 3 or more according to the method in Experiment 17 revealing that the enzyme preparation releases trehalose from .alpha.-glycosyltrehalose similarly as the trehalose-releasing enzyme derived from Rhizobium sp. M-11.

DEPR:
Among known microorganisms, those of the species Brevibacterium helvolum (ATCC 11822) and Micrococcus roseus (ATCC 186), which had been confirmed by the present inventors to produce the trehalose-releasing enzymes usable in the present invention, were respectively cultured by a fermenter at 27.degree. C. for 72 hours similarly as in Experiment 14. About 18 L of each resultant culture was similarly as in Experiment 15 subjected to a cell disrupter and centrifuged to obtain a supernatant which was then successively salted out with ammonium sulfate, dialyzed and subjected to an ion-exchange column to obtain a partially purified enzyme preparation, followed by studying the properties. The results are in Table 18 including those of Rhizobium sp. M-11 and Arthrobacter sp. Q36.

DEPR:
In accordance with the method in Experiment 25, the experiment to prepare trehalose by the partially purified enzyme preparations from non-reducing saccharides, having a trehalose structure as an end unit and a glucose polymerization degree of 3 or more, was conducted. As a result, it was revealed that similarly as the trehalose-releasing enzyme from Rhizobium sp. M-11, all the preparations release trehalose from .alpha.-glycosyltrehalose.

DEPR:
To prepare high trehalose content saccharide compositions from starch, the influence of the combination of enzymes and the liquefaction degrees of starch were studied. A 20% corn starch suspension was mixed with 0.1% calcium carbonate, d.s.b., and the mixture was adjusted to pH 6.5, mixed with 0.1-2.0% per g starch, d.s.b., of "TERMAMYL", .alpha.-amylase commercialized by Novo Industri A/S, Copenhagen, Denmark, enzymatically reacted at 95.degree. C. for 15 min, and autoclaved at 120.degree. C. for 10 min into a liquefied solution (DE 2.5-20.5). The resultant mixture was promptly cooled, and then mixed with 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme prepared by the method in Experiment 2, 10 units/g starch, d.s.b., of a purified trehalose-releasing enzyme prepared by the method in Experiment 15, 500 units/g starch, d.s.b., of a starch debranching isoamylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and 5 units/g starch, d.s.b., of a cyclomaltodextrin glucanotransferase specimen commercialized by Hayashibara Biochemical Laboratories, inc., Okayama, Japan, followed by the enzymatic reaction at pH 6.0 and 45.degree. C. for 24 hours. The reaction mixture was heated at 95.degree. C. for 10 min, cooled, mixed with 10 units/g starch, d.s.b., of glucoamylase, and enzymatically reacted at pH 5.0 for 10 hours. The reaction mixture thus obtained was analyzed on HPLC and examined for trehalose content (w/w %, d.s.b.) with respect to the total carbohydrates. As a control, a liquefied starch solution was first subjected to the action of the non-reducing saccharide-forming enzyme

and the trehalose-releasing enzyme, then the reaction mixture was similarly as above subjected to the action of glucoamylase, followed by examining the resultant mixture on HPLC. The results are in Table 19.

DEPR:
As is evident from the results in Table 19, it was revealed that in the case of preparing high **trehalose** content saccharide compositions from starch, a relatively-low liquefaction degree of starch, preferably, a DE less than 15, more preferably, a DE less than 10, is satisfactory. It was also found that, when the non-reducing saccharide-forming enzyme and the **trehalose**-releasing enzyme are used in combination with starch debranching enzyme and/or cyclomaltodextrin glucanotransferase, the **trehalose** yield from starch increases up to about 2-4 fold higher than that yielded by only using the former two enzymes. Therefore, the combination use is advantageous for an industrial-scale production of **trehalose** from starch.

DEPR:
Potato starch was prepared into an about 20 w/v % suspension which was then mixed with 0.3 w/v % oxalic acid and autoclaved, cooled and neutralized with calcium carbonate to obtain a liquefied solution with a pH of 6.5 and a DE of about 12. To the solution was added 2 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme and 300 units/g starch, d.s.b., of isoamylase, and enzymatically reacted at 45.degree. C. for 24 hours. The reaction mixture was heated to 95.degree. C. to inactivate the remaining enzyme, cooled and filtered to obtain a supernatant which was then in usual manner decolored with an activated charcoal, desalted and purified with ion-exchangers in H- and OH-form, and concentrated to obtain an about 50% syrup in a yield of about 90%, d.s.b. The product, a saccharide composition with a reduced reducibility which had a DE of about 8 and contained .alpha.-glycosyltrehalose and reducing amylaceous saccharides, was placed in an autoclave, mixed with 10% Raney nickel, and heated up to a temperature of 90.degree.-120.degree. C. while stirring, followed by increasing the hydrogen pressure to 20-120 kg/cm.sup.2 to terminate the hydrogenation. Thereafter, the Raney nickel was removed, and, in usual manner, decolored, desalted, purified and concentrated to obtain a 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Tapioca starch was prepared into an about 25% suspension which was then mixed with 0.2% per g starch, d.s.b., of "NEO-SPITASE", .alpha.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan. The resultant suspension was enzymatically reacted at 85.degree.-90.degree. C. for about 20 min, then autoclaved at 120.degree. C. and promptly cooled to obtain a liquefied solution with a DE of about 4. To the solution were added 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 9, 100 units/g starch, d.s.b., of pullulanase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and 20 units/g starch, d.s.b., of maltotetraose-forming enzyme produced by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and the mixture was enzymatically reacted at pH 6.5 and 40.degree. C. for 36 hours. The reaction mixture was similarly as in Example A-1 heated to inactivate the remaining enzyme, purified and concentrated into an about 60% solution. To increase the content of non-reducing saccharides in the solution, it was column chromatographed with "XT-1016", a strong-acid cation exchange resin in Ca.sup.2+ -form commercialized by Tokyo Organic Chemical Industries Ltd., Tokyo, Japan. The procedure was as follows: The resin was packed in 4 jacketed-stainless steel columns, having an inner diameter of 5.4 cm, which were then cascaded in series to give a total-gel-bed depth of 20 m. The columns were heated to give the inner column temperature of 55.degree. C., and fed with 5 v/v % of the solution as a feed solution while keeping at the temperature, followed by fractionating it by feeding to the columns with 55.degree. C. hot water at SV 0.2 to collect fractions rich in non-reducing

saccharides having a glucose polymerization degree of 4-6. The fractions thus obtained were pooled, purified and concentrated into an about 50% syrup, d.s.b. The syrup, a saccharide composition with a reduced reducibility and a DE 5.4 which contains .alpha.-glycosyltrehalose and reducing saccharides, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 50%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Corn starch was prepared into a 30% suspension which was then mixed with calcium carbonate to give the final concentration of 0.1%, d.s.b., and the resultant mixture was adjusted to pH 6.5, admixed with 0.3% per g starch, d.s.b., of "TERMAMYL 60L", .alpha.-amylase commercialized by Novo Industri A/S Copenhagen, Denmark, and subjected to an enzymatic reaction at 95.degree. C. for 15 min. The reaction mixture was autoclaved at 120.degree. C., promptly cooled into a liquefied solution (DE 4) which was then admixed with 4 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 2, 300 units/g starch, d.s.b., of isoamylase, and 5 units/g starch, d.s.b., of cyclomaltodextrin glucano-transferase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and subjected to an enzymatic reaction at pH 6.3 and 45.degree. C. for 48 hours. The reaction mixture was heated at 95.degree. C. for 10 min, cooled, admixed with 10 units/g starch, d.s.b., of .beta.-amylase, and enzymatically reacted at 55.degree. C. and pH 5.5 for 16 hours. The reaction mixture was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into an about 50% syrup. The syrup, a saccharide composition with a reduced reducibility which contained reducing saccharides and non-reducing saccharides such as those having a **trehalose** structure as an end unit and .alpha.-glycosyl .alpha.-glucosides, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability, and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
A syrup obtained by the method in Example A-3 was prepared into an about 55% solution which was then column chromatographed with a strong-acid cation exchange resin in alkaline form in accordance with the method in Example A-2, followed by collecting fractions rich in non-reducing saccharides with a glucose polymerization degree of 3-6. The fractions were pooled, purified and concentrated into an about 50% syrup. The syrup, a saccharide composition (DE 8) with a reduced reducibility which contained reducing amylaceous saccharides and non-reducing saccharides such as .alpha.-glycosyl .alpha.-glucosides and those having a **trehalose** structure as an end unit, was in accordance with the method in Example A-1 hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 30%, d.s.b. The syrup thus obtained is a saccharide composition with a reduced reducibility (DE of less than 1) which contains sugar alcohols and non-reducing saccharides having a **trehalose** structure within the molecules, and has a satisfactorily mild and high-quality sweetness, relatively-low viscosity, and adequate moisture-retaining ability. Thus, it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Corn starch was prepared into an about 30% suspension, and, in accordance with the method in Example A-3, .alpha.-amylase was allowed to act on the suspension

to obtain a liquefied solution (DE 4) which was then admixed with 5 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 2, 10 units/g starch, d.s.b., of a purified trehalose-releasing enzyme obtained by the method in Experiment 15, and 500 units/g starch, d.s.b., of isoamylase, and subjected to an enzymatic reaction at pH 6.0 and 40.degree. C. for 48 hours. The reaction mixture containing 76.3% trehalose, d.s.b., was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into an about 45% syrup. The syrup thus obtained was a non-reducing saccharide composition rich in trehalose, and, in accordance with the method in Example A-1, it was hydrogenated, purified and concentrated into an about 85% solution. The resultant solution was placed in a crystallizer, crystallized while gently stirring and gradually cooling, transferred to a plastic plain vessel, allowed to stand at ambient temperature for 2 days, and aged to terminate the crystallization and to form a block. The resultant block was pulverized by a cutter to obtain a powdery saccharide composition with a reduced reducibility containing hydrous trehalose and sugar alcohols in a yield of 80% with respect to the material starch, d.s.b. The product with a reduced reducibility (DE of less than 1) is readily handleable and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Tapioca starch was prepared into an about 30% suspension, and, in accordance with the method in Example A-2, .alpha.-amylase was allowed to act on the suspension to form a liquefied solution (DE 5), followed by adding to the solution 3 units/g starch, d.s.b., of a purified non-reducing saccharide-forming enzyme obtained by the method in Experiment 10, 5 units/g starch, d.s.b., of a purified trehalose-releasing enzyme obtained by the method in Experiment 23, and 200 units/g starch, d.s.b., of cyclomaltodextrin glucanotransferase, and subjecting the resultant mixture to an enzymatic reaction at 45.degree. C. for 48 hours. The reaction mixture containing 84.7% trehalose was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and continuously crystallized while concentrating. The resultant massecuite was separated by a basket-type centrifuge, and the resultant crystal was washed by spraying thereto a small amount of water to obtain a crystalline trehalose hydrate in a yield of about 55%, d.s.b. The resultant mother liquor, containing relatively-large amounts of reducing amylaceous saccharides, trehalose and non-reducing saccharide having a trehalose structure, was concentrated into a 50% syrup of saccharides with a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated, purified and concentrated to obtain a 70% syrup in a yield of about 30%, d.s.b. The product, a saccharide composition with a reduced reducibility (DE of less than 1) which contains trehalose, sugar alcohols and non-reducing saccharides having a trehalose structure, has a mild and high-quality sweetness, relatively-low viscosity, and satisfactory moisture-retaining ability. Thus, it can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stability, filler, excipient and/or diluent in a variety of compositions.

DEPR:
The heat inactivated reaction mixture in Example A-6 was mixed with 10 units/g substrate, d.s.b., of glucoamylase, and subjected to an enzymatic reaction at pH 5.0 and 50.degree. C. for 10 hours. The resultant reaction mixture was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated into a 45% syrup with a high trehalose content and a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated and purified into an about 70% solution which was then placed in a crystallizer and crystallized while stirring and gradually cooling to obtain a massecuite with a crystallization percentage of about 40%. The massecuite was sprayed at a pressure of 150 kg/cm.sup.2 from a nozzle mounted on the top of a drying tower while 85.degree. C. hot air was blowing to the contents from the upper part of the drying tower and collecting the resultant crystalline powders on a wire netting conveyer provided in the basement of the drying tower. The crystalline powders were gradually transferred out of the drying tower and recovered while 45.degree. C. hot air was blowing to the powders through under the conveyer. The crystalline powders

thus obtained were placed in an aging tower and aged for 10 hours while a hot air was blowing to the contents to terminate the crystallization and drying. Thus, a saccharide powder with a reduced reducibility, which contained hydrous trehalose crystal and sorbitol, was obtained in a yield of about 75% with respect to the material starch, d.s.b. The powder with a reduced reducibility (DE of less than 1) is readily handleable and can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and/or diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
A mutant of Rhizobium sp. M-11 (FERM BP-4130) was inoculated in a nutrient culture medium and cultured in a fermenter for about 70 hours in accordance with the method in Experiment 1. The resultant culture was filtered with an SF membrane to remove cells to obtain an about 100 L supernatant which was then concentrated with a UF membrane into an about 5 L enzyme concentrate containing about 410 units/ml of a non-reducing saccharide-forming enzyme and about 490 units/ml of a trehalose-releasing enzyme. Corn starch was prepared into an about 33% suspension which was then treated with .alpha.-amylase to obtain a liquefied solution (DE of about 4) in accordance with the method in Example A-3, mixed with 0.02 ml per g starch, d.s.b., of the concentrated enzyme solution, 500 units/g starch, d.s.b., of isoamylase, and 5 units/g starch, d.s.b., of cyclomaltodextrin glucanotransferase, and enzymatically reacted at pH 6.2 and 40.degree. C. for 48 hours. The reaction mixture was heated to inactivate the remaining enzyme, mixed with 10 units/g substrate, d.s.b., of glucoamylase, and enzymatically reacted at pH 5.0 and 50.degree. C. for 10 hours. The resultant mixture containing 85.6% trehalose, d.s.b., was heated to inactivate the remaining enzyme, and, in usual manner, decolored, desalted, purified and concentrated to obtain a 45% syrup of saccharides rich in trehalose with a reduced reducibility. In accordance with the method in Example A-1, the syrup was hydrogenated, purified, and, in accordance with the method in Example A-5, concentrated and crystallized to form a block which was then pulverized with a cutter to obtain a saccharide powder with a reduced reducibility, which contains hydrous trehalose crystal and sorbitol, in a yield of about 80% with respect to the material starch, d.s.b. The product with a reduced reducibility (DE of less than 1) is readily handleable and can be used in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Into a fermenter was poured a liquid nutrient culture medium consisting of 2 w/v % glucose, 0.5 w/v % polypeptone, 0.1 w/v % yeast extract, 0.1 w/v % dipotassium phosphate, 0.06 w/v % sodium dihydrogen phosphate, 0.05 w/v % magnesium sulfate, 0.5 w/v % calcium carbonate, and water, and the medium was sterilized by heating, cooled and inoculated with a seed culture of Pimelobacter sp. R48 (FERM BP-4315), followed by the incubation at 27.degree. C. for about 40 hours under stirring conditions. The resultant culture had 0.55 units/ml of a maltose-trehalose converting enzyme. 0.18 kg of wet cells collected from 18 L of the culture was suspended in 10 mM phosphate buffer (pH 7.0), and about 1.5 L of the suspension was treated with an ultrasonic cell disrupter to disrupt cells. The resultant mixture was centrifuged to obtain a supernatant which was then concentrated with a UF membrane to obtain an about 500 ml of a concentrated enzyme solution containing about 18 units/ml of a maltose-trehalose converting enzyme. To 15% corn starch suspension (pH 5.5) was added 2 units/g starch, d.s.b., of "SPITASE HS", .alpha.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and the mixture was stirred, heated to effect gelatinization and liquefaction, and then promptly autoclaved at 120.degree. C. for 20 min. Thereafter, the resultant mixture was cooled to 55.degree. C., adjusted to pH 5.0, mixed with 300 units/g starch, d.s.b., of isoamylase, 20 units/g starch, d.s.b., of .beta.-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and enzymatically reacted for 24 hours to obtain an about 92% maltose solution. The solution thus obtained was heated at 100.degree. C. for 20 min, cooled to 20.degree. C., adjusted to pH 7.0, and mixed with 1.5 units/g starch, d.s.b., of the concentrated enzyme solution prepared in the above, and enzymatically reacted for 72 hours. The resultant reaction mixture was heated at 95.degree. C. for 10 min, cooled, and, in usual manner, decolored with an activated charcoal, filtered, desalted and purified with ion-exchangers in H- and OH-form, and concentrated into an about 50% syrup.

DEPR:
The product contained about 64% **trehalose,** d.s.b., and had a low DE of 18.0. In accordance with the method in Example A-1, the syrup was hydrogenated, purified and concentrated to obtain an about 70% syrup in a yield of about 80%, d.s.b. The product, a saccharide syrup with a reduced reducibility (DE of less than 1) which contains **trehalose,** maltitol and a small amount of sorbitol, has a mild sweetness, adequate viscosity, and satisfactory moisture retaining ability, and these render it arbitrarily useful in a variety of compositions such as foods, cosmetics and pharmaceuticals.

DEPR:
Thirty-three parts by weight of a powdered orange juice prepared by spray drying was mixed to homogeneity under stirring conditions with 50 parts by weight of a powdery saccharide composition with a reduced reducibility obtained by the method in Example A-5, 10 parts by weight of sucrose, 0.65 parts by weight of anhydrous citric acid, 0.1 part by weight of malic acid, 0.1 part by weight of L-ascorbic acid, 0.1 part by weight of sodium citrate, 0.5 parts by weight of pullulan, and an adequate amount of a powdered flavor. The resultant mixture was pulverized, fed to a fluidized-bed granulator and granulated for 30 min by spraying to the contents a high **trehalose** content syrup, as a binder, obtained by the method in Example A-6 while 40.degree. C. air was sending to the contents. The granules thus obtained were weighed and packed to obtain the desired product. The product, a powdered juice containing about 30% orange juice, d.s.b., was stable for a relatively-long period of time without giving an unsatisfactory smell and taste.

DEPR:
Two hundred parts by weight of a powdery saccharide composition with a reduced reducibility, obtained by the method in Example A-5, and 300 parts by weight of maltose were mixed with 3 parts by weight of iodine dissolved in 50 parts by weight of methanol, and the resultant solution was admixed with 200 parts by weight of 10 w/v % pullulan solution to obtain the desired product with an adequate spreadability and adhesiveness. The product exerts a bactericidal activity due to the iodine and acts as an energy-supplementing agent for living cells due to the **trehalose,** and therefore, it can shorten the healing period and readily cure the wounded sites.

DEPR:
As is evident from above, the present saccharide composition, which comprises sugar alcohols and non-reducing saccharides consisting of **trehalose** and/or saccharides having a **trehalose** structure, has a satisfactory stability and a high-quality sweetness. The saccharide composition is assimilated, absorbed and utilized by the body when orally administered. More particularly, the **trehalose** contained in the saccharide composition is readily metabolized and utilized by the body. Thus, the present saccharide composition can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and diluent in a variety of compositions such as foods, cosmetics and pharmaceuticals. The reducing saccharides with a reduced reducibility used as a material for the present saccharide composition include (i) those prepared by allowing a non-reducing saccharide-forming enzyme together with a starch debranching enzyme and/or cyclomaltodextrin glucanotransferase to act on a liquefied starch solution whereby non-reducing saccharides such as **trehalose** and saccharides having a **trehalose** structure are formed in an increased yield to obtain the objective saccharide composition with a reduced reducibility and a relatively-low molecular weight and viscosity, and (ii) those prepared by allowing a maltose-**trehalose** converting enzyme to act on maltose to form a saccharide composition of maltose and **trehalose**. These saccharide compositions are satisfactorily used as a material for the present invention and facilitate the industrial-scale preparation of the present invention.

DEPL:
Production of **Trehalose**-Releasing Enzyme by Rhizobium sp. M-11

DEPL:
Property of **Trehalose**-Releasing Enzyme

DEPL:
Preparation of **Trehalose** by .alpha.-Glycosyltrehalose

DEPL:
Preparation of **Trehalose** from Reducing Partial Starch Hydrolysates

DEPL:
Production of **Trehalose**-Releasing Enzyme by Arthrobacter sp. Q36

DEPL:
Preparation of **Trehalose** from .alpha.-Glycosyltrehalose

DEPL:
Production and Property of **Trehalose**-Releasing Enzyme by known Microorganism

DEPL:
Influence of Starch Liquefaction Degree and Enzyme for Preparing High **Trehalose** Content Saccharide Composition

DEPV:
Specifically hydrolyzing the linkage between a **trehalose** moiety and other glycosyl moiety;

DEPV:
Converting maltose into **trehalose,** and vice versa;

DEPV:
1. The **trehalose**-releasing enzyme specifically hydrolyzes the linkage between a **trehalose** moiety and a glycosyl moiety in .alpha.-glycosyltrehalose to form **trehalose** and a reducing saccharide having a glucose polymerization degree of one or more; and

DEPV:
2. Maltooligosaccharide is not hydrolyzed by the **trehalose**-releasing enzyme.

DETL:
TABLE 5 ______________________________________ Saccharide Glucose **Trehalose** Molecular ratio preparation (%) (%) (Glucose/Trehalose) ______________________________________ P I 36.2 63.8 1.07 P II 52.0 48.0 2.06 P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11 ______________________________________

DETL:
TABLE 7 ______________________________________ Saccharide composition of hydrolysate by .alpha.-glucosidase Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________________ P I 36.5 63.0 0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3 0.3 ______________________________________

DETL:
TABLE 8 ______________________________________ Saccharide composition of hydrolysate by rat intestinal acetone powder Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________________ P I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P V 73.4 26.5 0.1 ______________________________________

DETL:
TABLE 13

____

Elution time Percentage Substrate Product on HPLC (min) (%)

____

Glucosyltrehalose **Trehalose** 27.4 17.5 Glucose 33.8 6.5 Glucosyltrehalose 23.3 76.0 Maltosyltrehalose **Trehalose** 27.4 44.3 Maltose 28.7 44.4 Maltosyltrehalose 21.6 11.3 Maltotriosyltrehalose **Trehalose** 27.4 39.5 Maltotriose 25.9 60.0 Maltotriosyltrehalose 19.7 0.5 Maltotetraosyltrehalose **Trehalose** 27.4 34.2 Maltotetraose 24.1 65.5 Maltotetraosyltrehalose 18.7 0.3 Maltopentaosyltrehalose **Trehalose** 27.4 29.1 Maltopentaose 22.6 70.6 Maltopentaosyltrehalose 17.8 0.3 Maltotriose Maltotriose 25.9 100

Maltotetraose Maltotetraose 24.1 100 Maltopentaose Maltopentaose 22.6 100 Maltohexaose Maltohexaose 21.8 100 Maltoheptaose Maltoheptaose 21.0 100

DETL:
TABLE 14 Glucose polymerization degree of reducing partial starch hydro- Composition (%) lysate Reaction product A B 34.1 **Trehalose** 80.8 83.5 Glucose 0.2 16.5 Reducing oligosaccharides 14.4 0.0 Glycosyltrehalose 4.6 0.0 26.2 **Trehalose** 79.7 82.5 Glucose 0.2 17.5 Reducing oligosaccharides 15.3 0.0 Glycosyltrehalose 4.8 0.0 18.1 **Trehalose** 77.7 80.7 Glucose 0.2 19.3 Reducing oligosaccharides 17.0 0.0 Glycosyltrehalose 5.1 0.0 15.2 **Trehalose** 75.0 78.5 Glucose 0.3 21.5 Reducing oligosaccharides 18.6 0.0 Glycosyltrehalose 6.1 0.0 10.0 **Trehalose** 66.1 70.1 Glucose 0.3 29.9 Reducing oligosaccharides 27.6 0.0 Glycosyltrehalose 7.7 0.0 3 **Trehalose** 4.2 20.8 (Maltotriose) Glucose 2.1 79.2 Maltotriose 65.0 0.0 Glucosyltrehalose 28.7 0.0 Note: In the Table, the symbol "A" means a composition after enzymatic reaction of a nonreducing saccharideforming enzyme and a trehalosereleasing enzyme and the symbol "B" means a composition after enzymatic reaction of glucoamylese. The wording "Glycosyltrehalose" means a nonreducing saccharide having a **trehalose** structure as an end unit and a glucose polymerization degree of 3 or more.

DETL:
TABLE 15 Coloration degree Saccharide preparation (480 nm) **Trehalose** (Present invention) 0.006 Glucose (Control) 1.671 Maltose (Control) 0.926

CLPR:
6. The food composition according to claim 5, wherein said saccharide composition contains **trehalose**.

CLPR:
10. The cosmetic composition according to claim 9, wherein said saccharide composition contains **trehalose**.

CLPR:
14. The pharmaceutical composition according to claim 13, wherein said saccharide composition contains **trehalose**.

CLPV:
comprising a sugar alcohol and a saccharide selected from the group consisting of **trehalose,** a non-reducing saccharide having a **trehalose** structure as an end unit, a non-reducing saccharide having a **trehalose** structure within the molecule, and a mixture thereof.

CLPV:
a sugar alcohol; a saccharide selected from the group consisting of a non-reducing saccharide having a **trehalose** structure as an end unit, a non-reducing saccharide having a **trehalose** structure within the molecule, and a mixture thereof; and,

CLPV:
optionally, **trehalose**.

CLPV:
a saccharide selected from the group consisting of a non-reducing saccharide having a **trehalose** structure as an end unit, a non-reducing saccharide having a **trehalose** structure within the molecule, and a mixture thereof; and

CLPV:
optionally, **trehalose**.

CLPV:
a saccharide selected from the group consisting of a non-reducing saccharide having a **trehalose** structure as an end unit, a non-reducing saccharide having a **trehalose** structure within the molecule, and a mixture thereof; and

CLPV:
optionally, **trehalose**.

CLPV:
a sugar alcohol and a saccharide selected from the group consisting of **trehalose**, a non-reducing saccharide having a **trehalose** structure as an end unit, a non-reducing saccharide having a **trehalose** structure within the molecule, and a mixture thereof; and,

ORPL:
Hoelzle, Inger and John G. Streeter; "Increased Accumulation of **Trehalose** in Rhizobia Cultured under 1% Oxygen"; Applied and Environmental Biology; vol. 56, No. 10; pp. 3213-3215; Oct. 1990.

USPT

US-CL-CURRENT: 435/100,536/102 ,536/123.1 ,536/127

US-PAT-NO: 5677442
DOCUMENT-IDENTIFIER: US 5677442 A
TITLE: Method of crystallizing **trehalose** without using organic solvent
DATE-ISSUED: October 14, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 536/123.13,435/100 ,536/102 ,536/123.1 ,536/127
ABSTRACT:

Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals. Also disclosed is a method of crystallizing **trehalose** from a 65 to 90% aqueous solution in the absence of organic solvent.

3 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

TTL:
Method of crystallizing **trehalose** without using organic solvent

ABPL:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield **trehalose** when allowed to act on the non-reducing saccharides. These non-reducing saccharides and **trehalose** are extensively useful in food products, cosmetics and pharmaceuticals. Also disclosed is a method of crystallizing **trehalose** from a 65 to 90% aqueous solution in the absence of organic solvent.

BSPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses, more particularly, to a novel non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, as well as to its preparation and microorganisms capable of producing said enzyme. The present invention further relates to a composition containing a non-reducing saccharide having a **trehalose** structure as an end unit which can be prepared with said enzyme, a relatively-low reducing saccharide containing said non-reducing saccharide, and/or **trehalose** prepared from these saccharides.

BSPR:
Trehalose or .alpha.,.alpha.-**trehalose** has long been known as a non-reducing saccharide consisting of glucose units. As described in Advances in Carbohydrate Chemistry, Vol. 18, pp. 201-225 (1963), published by Academic Press, U.S.A., and Applied and Environmental Microbiology, Vol. 56, pp. 3,213-3,215 (1990), **trehalose** widely exists in microorganisms, mushrooms, insects, etc., though the content is relatively low. In Table 2 of Advances in Carbohydrate Chemistry, Vol. 18, pp. 207 (1963), the physicochemical

properties, e.g. melting point and specific rotary power, of crystalline **trehalose** hydrate, i.e. crystalline .alpha.,.alpha.-**trehalose** dihydrate, which have been prepared from various natural sources, as reported in many publications, are listed. Since non-reducing saccharides including **trehalose** do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, non-reducing saccharides can be used with amino acids without causing browning and deterioration. Because of this, there has been great demand to establish a method for preparation of such a non-reducing saccharide.

BSPR:
In conventional preparations of **trehalose,** as disclosed in Japanese Patent Laid-Open No. 154,485/75, microorganisms are utilized, or as proposed in Japanese Patent Laid-Open No. 216,695/83, maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** present in microorganisms as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, unless specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (1) since **trehalose** is formed via glucose-1-phosphate, maltose as a substrate could not be used at a relatively-high concentration; (2) since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) it is substantially difficult to maintain the reactions systems stable and to continue their enzymatic reactions smoothly. Thus, there has not yet been realized an industrial-scale preparation of **trehalose**.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column entitled "Oligosaccharides" in the chapter entitled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No. 88, pp. 67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been reported to be scientifically almost impossible in this field." Thus, an enzymatic preparation of **trehalose** by using starch as a material has been deemed to be scientifically very difficult.

BSPR:
In order to attain the aforementioned object, the present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme, which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates.

BSPR:
As a result, we isolated novel microorganisms of the genera Rhizobium, named as "Rhizobium sp. M-11", and Arthrobacter, named as "Arthrobacter sp. Q36", from the respective soils in Okayama-city, Okayama, Japan, and in Soja-city, Okayama, Japan; and found that the microorganisms produce a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and that the objective non-reducing saccharides are readily prepared when the enzyme is allowed to act on reducing partial starch hydrolysates.

BSPR:
We also found that **trehalose** can be prepared by first allowing the enzyme to act on reducing partial starch hydrolysates, then subjecting the resultant non-reducing saccharides to the action of glucoamylase or .alpha.-glucosidase. Thus, the present inventors accomplished this invention. Also, we extensively screened microorganisms capable of producing the enzyme from conventional microorganisms.

BSPR:
As a result, it was found that microorganisms of the genera Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium and Terrabacter produce the present

non-reducing saccharide-forming enzyme as the microorganisms of the genera Rhizobium and Arthrobacter, and we accomplished this invention. Also, we established preparations of compositions such as food products, cosmetics and pharmaceuticals which contain the present non-reducing saccharides, relatively-low reducing saccharides containing the non-reducing saccharides and/or **trehalose** prepared from these saccharides, and accomplished this invention.

DEPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses. The present invention further relates to a microorganism capable of producing said enzyme, non-reducing saccharides prepared with said enzyme, relatively-low reducing saccharides containing said non-reducing saccharides, **trehalose** prepared from these saccharides, and compositions containing either or both of these saccharides and **trehalose**.

DEPR:
The present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and eventually found the objective microorganisms.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Rhizobium. The microorganism is similar to those of the species Rhizobium meliloti in some properties, but they are distinguishable with the fact that the present microorganism utilizes maltose, lactose and mannitol but forms no acid, and it produces a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such a microorganism having these properties.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol. 2 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Arthrobacter. The microorganism is characterized by producing a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such an enzyme.

DEPR:
The concentration of the reducing partial starch hydrolysates used as a substrate in the invention is not specifically restricted. While the present enzymatic reaction proceeds even with a 0.1% solution of a substrate, the enzymatic reaction more favorably proceeds with solutions having a concentration of 2% or higher, preferably, those having a concentration of 5-50% of a substrate, d.s.b. Under these concentrations non-reducing saccharides having a **trehalose** structure are readily formed in a satisfactorily-high yield. Suspensions containing insoluble substrates can be used in the invention. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 55.degree. C., preferably, a temperature in the range of 40.degree.-50.degree. C. The reaction pH used in the present enzymatic reaction is controlled in the range of 5-10, preferably, in the range of about 6-8. The reaction time used in the present enzymatic reaction is adequately chosen dependently on the conditions of the enzymatic reaction.

DEPR:
If necessary, the present non-reducing saccharides having a **trehalose** structure or relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase and .alpha.-glucosidase in order to control their sweetness and reducing power or to lower their viscosity; and the resultant products can be

further treated such that the remaining reducing saccharides are hydrogenated into sugar alcohols to diminish their reducing powder.

DEPR:
More particularly, **trehalose** is readily prepared by allowing glucoamylase or .alpha.-glucosidase to act on the present non-reducing saccharides or relatively-low reducing saccharides containing them. A high **trehalose** content fraction is obtained by allowing glucoamylase or .alpha.-glucosidase to act on these saccharides to form a mixture of **trehalose** and glucose, and subjecting the mixture to the aforementioned purifications such as ion-exchange column chromatography to remove glucose. The high **trehalose** content fraction can be arbitrarily purified and concentrated into a syrupy product, and, if necessary, the syrupy product can be concentrated into a supersaturated solution, followed by crystallizing hydrous- or anhydrous-crystalline **trehalose** and recovering the resultant crystal.

DEPR:
In order to prepare hydrous crystalline **trehalose,** an about 65-90% solution of **trehalose** with a purity of about 60% or higher is placed in a crystallizer, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10.degree.-90.degree. C., to obtain a massecuite containing hydrous crystalline **trehalose**. Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the invention to prepare from the massecuite hydrous crystalline **trehalose** or crystalline saccharides containing it.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline **trehalose** from the mother liquor, and, if necessary the hydrous crystalline **trehalose** is washed by spraying with a small amount of cold water to facilitate the preparation of hydrous crystalline **trehalose** with an increased purity. In the case of spray drying, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are really prepared by spraying massecuites with a concentration of 70-85%, d.s.b., and a crystallinity of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60.degree.-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto air heated to about 30.degree.-60.degree. C. In the case of block pulverization, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallinity of about 10-60%, d.s.b., to stand for about 0.1-3 days in order to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

DEPR:
Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into the anhydrous form, it is generally prepared by providing a concentrated solution of **trehalose** with a moisture content less than 10%; placing the solution in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50.degree.-160.degree. C., preferably, a temperature in the range of 80.degree.-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present non-reducing saccharides are hydrolyzed by amylases such as .alpha.-amylase derived from pancreas into relatively-low molecular weight non-reducing oligosaccharides or maltooligosaccharides, and these oligosaccharides are readily hydrolyzed by .alpha.-glucosidase and intestinal enzymes into glucose and **trehalose** molecules. The resultant **trehalose** is readily hydrolyzed by trehalase into glucoses. Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** can be utilized as an energy source by the body when orally

administered. These present saccharides and **trehalose** are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose**, have a satisfiable stability and sweetness, and those in crystalline form can be arbitrarily used as a sugar coating material for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinylpyrrolidone. These saccharides and **trehalose** have properties such as osmotic pressure-controlling ability, filler-imparting ability, gloss-imparting ability, moisture-retaining ability, viscosity-imparting ability, substantially no fermentability, ability to prevent retrogradation of gelatinized starch, and ability to prevent crystallization of other saccharides.

DEPR:
Anhydrous crystalline **trehalose** can be arbitrarily used as a desiccant for food products, cosmetics, pharmaceuticals, and their materials and intermediates, can be readily formed into compositions in the form of powder, granule and tablet with a satisfactory stability and quality.

DEPR:
Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient and desiccant in a variety of compositions such as food products, tobaccos, cigarettes, feeds, pet foods, cosmetics and pharmaceuticals.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, isomaltooligosaccharide, galactooligosaccharide, fructooligosaccharide, lactosucrose, sorbitol, maltitol, lactitol, dihydrocharcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as a powdery or crystalline **trehalose** prepared from these saccharides, can be used intact, or, if necessary they can be mixed with an excipient, filler and binder and formed into granules, spheres, shot-rods, plates, cubes and tablets, prior to their use.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides have the following features: (i) They have a sweetness which well harmonizes with other materials having sour-, acid-, salty-, bitter-, astringent- and delicious-tastes; and (ii) they are highly acid- and heat-resistant. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in seasonings such as amino acids, peptides, soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake), "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare"

(a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), nucleic acid condiments, mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
Also, the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be freely used for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker), "arare-mochi" (a rice-cake cube), "okoshi" (a millet-and-rice cake), "mochi" (a rice paste), "manju" (a bun with a bean-jam), "uiro" (a sweet rice jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly, pao de Castella and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickles and pickled products such as "fukujin-zuke" (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish ham, fish sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tenpura" (a Japanese deep-fat fried fish paste); "chinmi" (relish) such as "uni-no-shiokara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle), "saki-surume" (dried squid strips) and "fugu-no-mirin-boshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk products; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liquors; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix and "sokuseki-shiruco" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and beverages such as baby foods, foods for therapy, beverages supplemented with nutrition, peptide foods and frozen foods; as well as for improving the tastes and qualities of the aforementioned food-products.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be also used in feeds and pet foods for animals such as domestic animals, poultry, honey bees, silk worms and fishes in order to improve their taste preferences. These saccharides and **trehalose** can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent and stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used as a quality-improving agent and stabilizer in biologically active substances which may contain unstable effective ingredients and activities, as well as in health foods and pharmaceuticals containing the biologically active substances. Examples of such a biologically active substance are lymphokines such as .alpha.-, .beta.- and .gamma.-interferons, tumor necrosis factor-.alpha. (TNF-.alpha.), tumor necrosis factor-.beta. (TNF-.beta.), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2; hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox

vaccine, tetanus toxoid, Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. By using the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides, the aforementioned biologically active substances are arbitrarily prepared into health foods and pharmaceuticals with a satisfactorily-high stability and quality without a fear of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods to for incorporating the present non-reducing saccharides, relatively-low reducing saccharides containing them and/or **trehalose** prepared from these saccharides into the above-mentioned compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. These saccharides and **trehalose** are usually incorporated into the above-mentioned compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
Fifty mg aliquots of non-reducing saccharides P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for 6 hours. The only saccharides detected in every resultant mixture on HPLC analysis were glucose and **trehalose**. The contents of the detected glucose and **trehalose**, and their molecular ratios were as shown in Table 5.

DEPR:
As evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one mole of glucose and one mole of **trehalose**; P II, hydrolyzed into two moles of glucose and one mole of **trehalose**; (iii) P III, hydrolyzed into three moles of glucose and one mole of **trehalose**; (iv) P IV, hydrolyzed into four moles of glucose and one mole of **trehalose**; and (v) P V, hydrolyzed into five moles of glucose and one mole of **trehalose**.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more moles of glucose bound to one mole of **trehalose** via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing saccharide having a degree of glucose polymerization of 3 (DP 3) and consisting of one mole of glucose bound to one mole of **trehalose**; P II, a non-reducing saccharide having DP 4 and consisting of two moles of glucose bound to one mole of **trehalose**; P III, a non-reducing saccharide having DP 5 and consisting of three moles of glucose bound to one mole of **trehalose**; P IV, a non-reducing saccharide having DP 6 and consisting of four moles of glucose bound to one mole of **trehalose**; and P V, a non-reducing saccharide having DP 7 and consisting of five moles of glucose bound to one mole of **trehalose**. It was revealed that, when .beta.-amylase acted on these non-reducing saccharides similarly as with glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one mole of maltose and one mole of P I, one mole of maltose and one mole of P II, and two moles of maltose and one mole of P I.

DEPR:
Based on these results, it was concluded that the enzymatic reaction of the present non-reducing saccharide-forming enzyme is an intramolecular reaction that occurs without changing the molecular weights of the substrates used, i.e. an intramolecular reaction that occurs without changing their degrees of glucose polymerization. It was concluded that the non-reducing saccharides P

I, P II, P III, P IV and P V were the respective .alpha.-glycosyl trehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.-**trehalose** residue) of .alpha.-glucosyl **trehalose,** .alpha.-maltosyl **trehalose,** .alpha.-maltotriosyl **trehalose,** .alpha.-maltotetraosyl **trehalose** and .alpha.-maltopentaosyl **trehalose**.

DEPR:
As evident from the results in Tables 7 and 8, it was revealed that similarly as in Experiment 6 with glucoamylase the saccharide preparations P I, P II, P III, P IV and P V were hydrolyzed by .alpha.-glucosidase and rat intestinal acetone powder into glucose and **trehalose** molecules.

DEPR:
To the resultant hydrolysate obtained with .alpha.-glucosidase or rat intestinal acetone powder was added one unit trehalase derived from pig kidney, an enzyme preparation of Sigma Chemical Company, St., Louis, U.S.A., and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC to reveal that **trehalose,** formed from the saccharide preparations P I, P II, P III, P IV and P V, was hydrolyzed by trehalase into glucose molecules.

DEPR:
Based on these results, it was concluded that the present non-reducing saccharide-forming enzyme is a novel enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates to a non-reducing end unit, a **trehalose** residue, i.e. a **trehalose** structure.

DEPR:
By using the purified enzyme preparation obtained in Experiment 10, the preparation and the confirmation of the structure of non-reducing saccharides were conducted in accordance with the methods in Experiments 4 and 6. As a result, it was revealed that the enzyme preparation forms one or more non-reducing saccharides, which saccharide has a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher, when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
In accordance with the method in Experiment 12, non-reducing saccharides were prepared by using partially purified enzyme preparations from these known microorganisms, and their structures were studied to find that, similarly as the non-reducing saccharide-forming enzyme from Rhizobium sp. M-11, every enzyme preparation formed non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose**; and Examples B illustrate compositions containing one or more of these saccharides and **trehalose**.

DEPR:
The saccharide solution thus obtained contained 29.5% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 90% **trehalose,** d.s.b. The fraction was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with about 2%, d.s.b., hydrous crystalline **trehalose** as a seed crystal and gradually cooled to obtain a massecuite with a degree of crystallization of about 45%. The massecuite was sprayed from a nozzle equipped on the top of a spraying tower at a pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air sent from the top of the spraying tower, and the resultant

crystalline powder was collected on a metal wire netting conveyer provided on the basement of the spraying tower, and gradually moved out of the tower while a stream of 40.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an ageing tower and aged for 10 hours to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline **trehalose**.

DEPR:
In accordance with the method in Example A-3, 30% suspension of corn starch was subjected to the action of an .alpha.-amylase specimen commercialized by Novo Industri A/S, Copenhagen, Denmark; a maltotetraose forming amylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan; and an .alpha.-amylase specimen commercialized by Ueda Chemical Co., Ltd., Osaka, Japan. The resultant mixture was autoclaved at 120.degree. C., cooled to 45.degree. C., admixed with 2 units per g starch of a non-reducing saccharide-forming enzyme prepared by the above-mentioned method, and subjected to an enzymatic reaction for 64 hours. The reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme. In accordance with the method in Example A-6, the resultant solution was subjected to the action of glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, decolored, desalted and concentrated into an about 60% solution. The saccharide solution thus obtained contained about 25% **trehalose,** d.s.b. The saccharide solution was fractionated on column chromatography using a strongly-acidic cation-exchange resin to obtain fractions rich in **trehalose**. The fractions were pooled, placed in a vessel and boiled down under a reduced pressure into a syrup with a moisture content of about 4.0%. The syrup was placed in a crystallizer and admixed with one % of anhydrous crystalline **trehalose,** as a seed crystal, with respect to the syrup, d.s.b., followed by crystallizing anhydrous crystalline **trehalose** at 95.degree. C. for 5 min while stirring. The resultant was transferred to an aluminum container and aged at 100.degree. C. for 6 hours to form a block. The resultant block was pulverized by a cutting machine and subjected to a fluidized-bed drying to obtain a powdery anhydrous crystalline **trehalose** with a moisture content of about 0.3%.

DEPR:
According to the method in Example A-6, 40 parts by weight of a partial starch hydrolysate was dissolved by heating in 60 parts by weight of water, and the solution was successively subjected to the action of a non-reducing saccharide-forming enzyme and glucoamylase to obtain a reaction mixture containing 29.5 w/w % **trehalose,** d.s.b. The reaction mixture was decolored, desalted, concentrated, and fed to column chromatography to obtain an about 90 w/w % **trehalose** solution.

DEPR:
The **trehalose** solution was concentrated into an about 75 w/w % solution, transferred to a crystallizer, mixed with about 2 w/w %, d.s.b., of crystalline **trehalose** hydrate as a seed crystal, and gradually cooled to obtain a massecuite with a crystallization percentage of about 45 w/w %, d.s.b., which was then separated on a basket-type centrifuge. The crystalline **trehalose** hydrate thus obtained was washed by spraying thereto a small amount of cold water to obtain a high-purity crystalline **trehalose** hydrate in a yield of about 10 w/w %, d.s.b.

DEPR:
The purity of the crystalline **trehalose** hydrate was about 99.6 w/w %, d.s.b., the melting point was about 97.degree. C., and the specific rotation was [.alpha.].sub.20.sup.D +180.degree. (c=5, H.sub.2 O). The specific rotation of crystalline **trehalose** anhydride was [.alpha.].sub.20.sup.D +199.degree. (c=5, H.sub.2 O).

DEPR:
An about 90 w/w %, d.s.b., of **trehalose** rich solution obtained by the method in Example A-9 was filtered with a UF-membrane having a molecular weight cut-off 6,000 daltons. The filtrate was concentrated into an about 74 w/w % solution which was then placed in a crystallizer, admixed with an about one w/w % of a crystalline **trehalose** hydrate as a seed crystal, and gradually cooled to obtain a massecuite with a crystallization percentage of about 43 w/w %, d.s.b.

Similarly as in Example A-9, the massecuite was separated on a basket-type centrifuge and washed to obtain a high-purity crystalline **trehalose** hydrate in a yield of about 8 w/w %, d.s.b.

DEPR:
The crystalline **trehalose** hydrate is substantially free of hygroscopicity and readily handleable, and these render it arbitrarily useful in a variety of compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, stabilizer, filler, excipient and adjuvant.

DEPR:
Three parts by weight of gum base was melted by heating until it softened, and the resultant was mixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method of Example A-6, and further mixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was kneaded by a roll in the usual manner, formed and packed to obtain the desired product.

DEPR:
Forty % "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was mixed with 2 parts by weight of a powder containing hydrous crystalline **trehalose** prepared by the method of Example A-6, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfactory taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:
Egg yolks prepared from fresh eggs were sterilized at 60.degree.-64.degree. C. by a plate heater, and the resultant liquid was mixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method of Example A-8 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was permitted to hydrate to hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method of Example A-6, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose**, one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to obtain a sugar coated tablet having a satisfactory gloss and appearance.

DEPR:
As evident from above, the present novel non-reducing saccharide-forming enzyme converts reducing partial starch hydrolysates into non-reducing saccharides in a satisfactorily-high yield under a relatively-mild enzymatic reaction condition without changing the degrees of glucose polymerization of the reducing partial starch hydrolysates. The non-reducing saccharides, which can be readily separated and purified, and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, have a satisfactory stability, quality and mild sweetness. These products are assimilated and utilized as an energy source by the body when orally administered. These non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be arbitrarily used in compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thus, the present invention provides a novel technique for preparing on an

industrial-scale and at a relatively-low cost non-reducing saccharides, which could not have been readily obtained in spite of their great demands, by using reducing partial starch hydrolysates prepared from starch as a cheap and abundant source, as well as to prepare relatively-low reducing saccharides containing the non-reducing saccharides, and **trehalose** prepared from these saccharides. The present invention has a great influence on the fields such as starch-, enzyme- and biochemical-sciences; and other industrial fields, especially, food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomable.

DEPV:
Forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

DEPV:
(1) The present non-reducing saccharide-forming enzyme forms non-reducing saccharides having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher without changing their degrees of glucose polymerization; and

DEPV:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is hydrolyzed by glucoamylase into one mole of **trehalose** and two moles of glucose.

DETL:
Carbon source Utilization Acid
formation D-Glucose + + D-Galactose +
+ D-Fructose + + L-Arabinose + + D-Xylose + + L-Rhamnose + + Maltose + -
Sucrose + + Lactose + - **Trehalose** + - Raffinose + + Mannitol + - Dextrin +
- Dulcitol + -

DETL:
TABLE 5 Saccharide Glucose **Trehalose**
Molecular ratio preparation (%) (%) (Glucose/Trehalose)
P I 36.2 63.8 1.07 P II 52.0 48.0 2.06
P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11

DETL:
TABLE 7 Saccharide composition of
hydrolysate with .alpha.-glucosidase Glucose **Trehalose** Other saccharides
Saccharide (%) (%) (%) P I 36.5 63.0
0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3
0.3

DETL:
TABLE 8 Saccharide composition of
hydrolysate with rat intestinal acetone powder Glucose **Trehalose** Other
saccharides Saccharide (%) (%) (%) P
I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P
V 73.4 26.5 0.1

CLPR:
1. A method for purifying **trehalose,** which comprises;

CLPR:
2. The method as claimed in claim 1, wherein said aqueous **trehalose** solution is prepared with a partial hydrolysate of a reducing amylaceous saccharide.

CLPR:
3. The method as claimed in claim 1, wherein the temperature for crystallizing **trehalose** in step (b) is not greater than 95.degree. C.

CLPV:

(a) providing an aqueous **trehalose** solution free of organic solvent;

CLPV:
(b) concentrating the aqueous **trehalose** solution into an about 65-90% aqueous solution;

CLPV:
(c) crystallizing **trehalose** in said aqueous solution of step (b); and

CLPV:
(d) separating and collecting the crystallized crystalline **trehalose** hydrate.

ORPL:
Database WPI, Section Ch, Week 9425, Derwent Publications Lt.d, London, GB; Class D16, AN 94-206435; "Alpha, alpha**-trehalose** production comprising extraction from yeast and water, and treatment with ultrafiltration or reverse osmosis"; and JP,A, 06 145 186 (Kirin Brewer KK and Nippon Shokuhin Kako KK), 24 May 1994, Abstract.

ORPL:
Hoelzle et al., "Increased Accumulation of **Trehalose** in Rhizobia Cultured under 1% Oxygen," Applied and Environmental Microbiology, pp. 3213-3215, Oct. 1990.

ORPL:
Journal of the Chemical Society, May 1965 Letchworth, GB, pp. 3489-3490, Birch, "A method of obtaining crystalline anhydrous alphaalpha**-trehalose**".

ORPL:
Biotechnology Letters, vol. 12, No. 6, Jun. 1990, pp. 431-432; Lama et al; "Starch conversion with immobilized thermophilic Archaebacterium **Sulfolobus** solfataricus".

USPT

US-CL-CURRENT: 435/100,435/101 ,435/105 ,435/200 ,435/209 ,435/252.5 ,435/278

US-PAT-NO: 5658765
DOCUMENT-IDENTIFIER: US 5658765 A
TITLE: Xylanase process for producing the same method for the treatment of pulp and production of xylo-oligosaccharides
DATE-ISSUED: August 19, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Noguchi; Yoshitaka	Chiba	N/A	N/A	JPX
Ikeda; Kazuko	Chiba	N/A	N/A	JPX
Masatsuji; Eiko	Chiba	N/A	N/A	JPX
Seko; Masahiko	Chiba	N/A	N/A	JPX

US-CL-CURRENT: 435/99,435/100 ,435/101 ,435/105 ,435/200 ,435/209 ,435/252.5 ,435/278
ABSTRACT:
Disclosed are novel xylanases, a process for producing the enzyme, a microorganism capable of producing the enzyme, a method for the treatment of pulp with the xylanase enzyme, and a process for producing xylose or xylo-oligosaccharide using the enzyme.
6 Claims, 4 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 4

DEPR:
Further in view of the fact that the strain grows at temperatures above 55.degree. C., it is considered that the strain is akin to B. stearothermophilus, B. schelegelii, B. acidocaldarius, B. licheniformis, B. coagulans, B. brevis, etc. However, the properties of anaerobic growth, VP test, VP broth pH and growth temperature range shown in Table 3 below reveal that strain SD902 has bacteriological properties dissimilar to any of these known standard bacteria belonging to the genus Bacillus.

DETL:
1) L-arabinose .+-. 2) D-xylose + 3) D-glucose + 4) D-mannose + 5) D-fructose + 6) D-galactose - 7) maltose + 8) sucrose .+-. 9) lactose + 10) trehalose .+-. 11) D-sorbitol + 12) D-mannitol + 13) inositol + 14) glycerine + 15) starch -

USPT :

US-CL-CURRENT: 435/200,435/95 ,435/96 ,435/97 ,435/99

US-PAT-NO: 5610047
DOCUMENT-IDENTIFIER: US 5610047 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: March 11, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
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ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.
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ABPL:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.

BSPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, its preparation and uses, more particularly, to a novel non-reducing saccharide-forming enzyme which forms a non-reducing saccharide having a trehalose structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher, as well as to its preparation and microorganisms capable of producing said enzyme. The present invention further relates to a composition containing a non-reducing saccharide having a trehalose structure as an end unit which is preparable with said enzyme, a relatively-low reducing saccharide containing said non-reducing saccharide, and/or trehalose prepared from these saccharides.

BSPR:
Trehalose or .alpha., .alpha.-trehalose has long been known as a non-reducing saccharide consisting of glucose units. As described in Advances in Carbohydrate Chemistry, Vol. 18, pp. 201-225 (1963), published by Academic Press, USA, and Applied and Environmental Microbiology, Vol. 56, pp. 3,213-3,215 (1990), trehalose widely exists in microorganisms, mushrooms, insects, etc., though the content is relatively low. Since non-reducing saccharides including trehalose do not react with substances containing amino groups such as amino acids and proteins, they neither induce the amino-carbonyl reaction nor alter amino acid-containing substances. Thus, non-reducing saccharides can be used with amino acids without causing browning and deterioration. Because of this has been in great demand to establish a method for preparation of such a non-reducing saccharide.

BSPR:
In conventional preparations of **trehalose,** as disclosed in Japanese Patent Laid-Open No. 154,485/75, microorganisms are utilized, or as proposed in Japanese Patent Laid-Open No. 216,695/83, maltose is converted into **trehalose** by using maltose- and **trehalose**-phosphorylases in combination. The former, however, is not suitable for industrial-scale preparation because the content of **trehalose** present in microorganisms as a starting material is usually lower than 15 w/w % (the wording "w/w %" will be abbreviated as "%" in the specification, if specified otherwise), on a dry solid basis (d.s.b.), and the extraction and purification steps are complicated. The latter has the following demerits: (i) Since **trehalose** is formed via glucose-1-phosphate, maltose as a substrate could not be used at a relatively-high concentration; (ii) Since the enzymatic reaction systems of the phosphorylases are reversible reactions, the yield of the objective **trehalose** is relatively low; and (iii) it is substantially difficult to maintain the reaction systems stably and to continue their enzymatic reactions smoothly. Thus, there has not yet been realized an industrial-scale preparation of **trehalose**.

BSPR:
As regards the preparation of **trehalose,** it is reported in the column titled "Oligosaccharides" in the chapter titled "Current Status of Starch Application Development and Related Problems" in "Food Chemicals", No. 88, pp. 67-72 (August, 1992) that "In spite of a wide applicability of **trehalose,** an enzymatic preparation thereof via a direct saccharide-transfer reaction or a hydrolytic reaction has been reported to be scientifically almost impossible in this field." Thus, an enzymatic preparation of **trehalose** by using starch as a material has been deemed to be scientifically very difficult.

BSPR:
In order to attain the aforementioned object, the present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme, which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates.

BSPR:
As a result, we isolated novel microorganisms of the genera Rhizobium, named as "Rhizobium sp. M-11", and Arthrobacter, named as "Arthrobacter sp. Q36", from the respective soils in Okayama-city, Okayama, Japan, and in Soja-city, Okayama, Japan; and found that the microorganisms produce a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and that the objective non-reducing saccharides are readily prepared when the enzyme is allowed to act on reducing partial starch hydrolysates.

BSPR:
We also found that **trehalose** can be prepared by first allowing the enzyme to act on reducing partial starch hydrolysates, then subjecting the resultant non-reducing saccharides to the action of glucoamylase or .alpha.-glucosidase. Thus, the present inventors accomplished this invention. Also, we extensively screened microorganisms capable of producing the enzyme from conventional microorganisms.

BSPR:
As a result, it was found that microorganisms of the genera Brevibacterium, Flavobacterium, Micrococcus, Curtobacterium and Terrabacter produce the present non-reducing saccharide-forming enzyme as the microorganisms of the genera Rhizobium and Arthrobacter, and we accomplished this invention. Also, we established preparations of compositions such as food products, cosmetics and pharmaceuticals which contain the present non-reducing saccharides, relatively-low reducing saccharides containing the non-reducing saccharides and/or **trehalose** prepared from these saccharides, and accomplished this invention.

DEPR:
The present invention relates to a novel non-reducing saccharide-forming enzyme, and its preparation and uses. The present invention further relates to

a microorganism capable of producing said enzyme, non-reducing saccharides prepared with said enzyme, relatively-low reducing saccharides containing said non-reducing saccharides, **trehalose** prepared from these saccharides, and compositions containing either or both of these saccharides and **trehalose**.

DEPR:
The present inventors have extensively screened microorganisms capable of producing a novel non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates, and eventually found the objective microorganisms.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol. 1 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Rhizobium. The microorganism is similar to those of the species Rhizobium meliloti in some properties, but they are distinguishable with the fact that the present microorganism utilizes maltose, lactose and mannitol but forms no acid, and it produces a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such a microorganism having these properties.

DEPR:
The bacteriological properties were compared with those of known microorganisms with reference to Bergey's Manual of Systematic Bacteriology, Vol. 2 (1984). As a result, it was revealed that the microorganism was identified as a microorganism of the genus Arthrobacter. The microorganism is characterized by producing a non-reducing saccharide-forming enzyme which forms non-reducing saccharides having a **trehalose** structure when allowed to act on reducing partial starch hydrolysates. No publications have reported such an enzyme. Based on these results, the present inventors named this microorganism "Arthrobacter sp. Q36", and deposited it on Jun. 3, 1993, in National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Ibaraki, Japan. The deposition of the microorganism was accepted on the same day and has been maintained by the institute under the accession number of FERM BP-4316.

DEPR:
Forming non-reducing saccharides having a **trehalose** structure as an end unit when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher;

DEPR:
The concentration of the reducing partial starch hydrolysates used as a substrate in the invention is not specifically restricted. While the present enzymatic reaction proceeds even with a 0.1% solution of a substrate, the enzymatic reaction more favorably proceeds with solutions having a concentration of 2% or higher, preferably, those having a concentration of 5-50% of a substrate, d.s.b. Under these concentrations non-reducing saccharides having a **trehalose** structure are readily formed in a satisfactorily-high yield. Suspensions containing insoluble substrates can be used in the invention. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the present enzyme is not inactivated, i.e. a temperature up to about 55.degree. C., preferably, a temperature in the range of 40.degree.-50.degree. C. The reaction pH used in the present enzymatic reaction is controlled in the range of 5-10, preferably, in the range of about 6-8. The reaction time used in the present enzymatic reaction is adequately chosen dependently on the conditions of the enzymatic reaction.

DEPR:
If necessary, the present non-reducing saccharides having a **trehalose** structure or relatively-low reducing saccharides containing the non-reducing saccharides can be hydrolyzed by amylases such as .alpha.-amylase, .beta.-amylase, glucoamylase and .alpha.-glucosidase in order to control their sweetness and reducing power or to lower their viscosity; and the resultant products can be

further treated such that the remaining reducing saccharides are hydrogenated into sugar alcohols to diminish their reducing powder.

DEPR:
More particularly, **trehalose** is readily prepared by allowing glucoamylase or .alpha.-glucosidase to act on the present non-reducing saccharides or relatively-low reducing saccharides containing them. A high **trehalose** content fraction is obtained by allowing glucoamylase or .alpha.-glucosidase to act on these saccharides to form a mixture of **trehalose** and glucose, and subjecting the mixture to the aforementioned purifications such as ion-exchange column chromatography to remove glucose. The high **trehalose** content fraction can be arbitrary purified and concentrated into a syrupy product, and, if necessary the syrupy product can be concentrated into a supersaturated solution, followed by crystallizing hydrous- or anhydrous-crystalline **trehalose** and recovering the resultant crystal.

DEPR:
In order to prepare hydrous crystalline **trehalose**, an about 65-90% solution of **trehalose** with a purity of about 60% or higher is placed in a crystallizer, and gradually cooled while stirring in the presence of 0.1-20% seed crystal at a temperature of 95.degree. C. or lower, preferably, at a temperature in the range of 10.degree.-90.degree. C., to obtain a massecuite containing hydrous crystalline **trehalose**. Conventional methods such as separation, block pulverization, fluidized-bed granulation and spray drying can be employed in the invention to prepare from the massecuite hydrous crystalline **trehalose** or crystalline saccharides containing it.

DEPR:
In the case of separation, massecuites are usually subjected to a basket-type centrifuge to separate hydrous crystalline **trehalose** from the mother liquor, and, if necessary the hydrous crystalline **trehalose** is washed by spraying with a small amount of cold water to facilitate the preparation of hydrous crystalline **trehalose** with an increased purity. In the case of spray drying, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by spraying massecuites with a concentration of 70-85%, d.s.b., and a crystallinity of about 20-60%, d.s.b., from a nozzle by a high-pressure pump; drying the resultant products with a 60.degree.-100.degree. C. hot air which does not melt the resultant crystalline powders; and aging the resultant powders for about 1-20 hours while blowing thereto a air heated to about 30.degree.-60.degree. C. In the case of block pulverization, crystalline saccharides with no hygroscopicity or which are substantially free of hygroscopicity are readily prepared by allowing massecuites with a moisture content of 10-20% and a crystallinity of about 10-60%, d.s.b., to stand for about 0.1-3 days in order to crystallize and solidify the whole contents into blocks; and pulverizing or cutting the resultant blocks.

DEPR:
Although anhydrous crystalline **trehalose** can be prepared by drying hydrous crystalline **trehalose** to convert it into the anhydrous form, it is generally prepared by providing a concentrated solution of **trehalose** with a moisture content less than 10%; placing the solution in a crystallizer; keeping the solution in the presence of a seed crystal at a temperature in the range of 50.degree.-160.degree. C., preferably, a temperature in the range of 80.degree.-140.degree. C. under stirring conditions to obtain a massecuite containing anhydrous crystalline **trehalose**; and crystallizing and pulverizing anhydrous crystalline **trehalose** by conventional methods such as block pulverization, fluidized-bed granulation and spray drying.

DEPR:
The present non-reducing saccharides are hydrolyzed by amylases such as .alpha.-amylase derived from pancreas into relatively-low molecular weight non-reducing oligosaccharides or maltooligosaccharides, and these oligosaccharides are readily hydrolyzed by .alpha.-glucosidase and intestinal enzymes into glucose and **trehalose** molecules. The resultant **trehalose** is readily hydrolyzed by trehalase into glucoses. Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose**, can be utilized as an energy source by the body when orally

administered. These present saccharides and **trehalose** are not substantially fermented by dental carries-inducing microorganisms, and this renders them useful as a dental carries-preventing sweetener.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose,** have a satisfiable stability and sweetness, and those in crystalline form can be arbitrarily used as a sugar coating material for tablets in combination with binders such as pullulan, hydroxyethyl starch and polyvinylpyrrolidone. These saccharides and **trehalose** have properties such as osmotic pressure-controlling ability, filler-imparting ability, gloss-imparting ability, moisture-retaining ability, viscosity-imparting ability, substantial no fermentability, ability to prevent retrogradation of gelatinized starch, and ability to prevent crystallization of other saccharides.

DEPR:
Anhydrous crystalline **trehalose** can be arbitrarily used as a desiccant for food products, cosmetics, pharmaceuticals, and their materials and intermediates, and can readily be formed into compositions in the form of powder, granule and tablet with a satisfactory stability and quality.

DEPR:
Thus, the present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, excipient and desiccant in a variety of compositions such as food products, tobaccos, cigarettes, feeds, pet foods, cosmetics and pharmaceuticals.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, can be used intact as a seasoning for sweetening. If necessary, they can be used together with adequate amounts of one or more other sweeteners, for example, powdered syrup, glucose, maltose, sucrose, isomerized sugar, honey, maple sugar, isomaltooligosaccharide, galactooligosaccharide, fructooligosaccharide, lactosucrose, sorbitol, maltitol, lactitol, dihydrocharcone, stevioside, .alpha.-glycosyl stevioside, rebaudioside, glycyrrhizin, L-aspartyl L-phenylalanine methyl ester, saccharin, glycine and alanine; and/or a filler such as dextrin, starch and lactose.

DEPR:
The present non-reducing saccharides and relatively-low reducing saccharides containing them, as well as a powdery or crystalline **trehalose** prepared from these saccharides, can be used intact, or, if necessary they can be mixed with an excipient, filler and binder and formed into granules, spheres, shot-rods, plates, cubes and tablets, prior to their use.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides have the following features: (i) They have a sweetness which well harmonizes with other materials having sour- , acid-, salty-, bitter-, astringent- and delicious-tastes; and (ii) they are highly acid- and heat-resistant. Thus, they can be favorably used in food products in general as a sweetener, taste-improving agent and quality-improving agent.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used in seasonings such as amino acids, peptides, soy sauce, powdered soy sauce, "miso", "funmatsu-miso" (a powdered miso), "moromi" (a refined sake), "hishio" (a refined soy sauce), "furikake" (a seasoned fish meal), mayonnaise, dressing, vinegar, "sanbai-zu" (a sauce of sugar, soy sauce and vinegar), "funmatsu-sushi-su" (powdered vinegar for sushi), "chuka-no-moto" (an instant mix for Chinese dish), "tentsuyu" (a sauce for Japanese deep-fat fried food), "mentsuyu" (a sauce for Japanese vermicelli), sauce, catsup, "yakiniku-no-tare"

(a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "dashi-no-moto" (an instant stock mix), nucleic acid condiments, mixed seasoning, "mirin" (a sweet sake), "shin-mirin" (a synthetic mirin), table sugar and coffee sugar.

DEPR:
Also, the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be freely used for sweetening "wagashi" (Japanese cakes) such as "senbei" (a rice cracker), "arare-mochi" (a rice-cake cube), "okoshi" (a millet-and-rice cake), "mochi" (a rice paste), "manju" (a bun with a bean-jam), "uiro" (a sweet rice jelly), "an" (a bean jam), "yokan" (a sweet jelly of beans), "mizu-yokan" (a soft adzuki-bean jelly), "kingyoku" (a kind of yokan), jelly, pao de Castella and "amedama" (a Japanese toffee); confectioneries such as bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel and candy; frozen desserts such as ice cream and sherbet; syrups such as "kajitsu-no-syrup-zuke" (a preserved fruit) and "korimitsu" (a sugar syrup for shaved ice); pastes such as flour paste, peanut paste, fruit paste and spread; processed fruits and vegetables such as jam, marmalade, "syrup-zuke" (fruit pickles) and "toka" (conserves); pickles and pickled products such as "fukujin-zuke" (red colored radish pickles), "bettara-zuke" (a kind of whole fresh radish pickles), "senmai-zuke" (a kind of sliced fresh radish pickles) and "rakkyo-zuke" (pickled shallots); premixes for pickles and pickled products such as "takuan-zuke-no-moto" (a premix for pickled radish) and "hakusai-zuke-no-moto" (a premix for fresh white rape pickles); meat products such as ham and sausage; products of fish meat such as fish ham, fish sausage, "kamaboko" (a steamed fish paste), "chikuwa" (a kind of fish paste) and "tenpura" (a Japanese deep-fat fried fish paste); "chinmi" (relish) such as "uni-no-shiokara" (salted guts of sea urchin), "ika-no-shiokara" (salted guts of squid), "su-konbu" (processed tangle), "saki-surume" (dried squid strips) and "fugu-no-mirin-boshi" (a dried mirin-seasoned swellfish); "tsukudani" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, fish and shellfish; daily dishes such as "nimame" (cooked beans), potato salad and "konbu-maki" (a tangle roll); milk producs; canned and bottled products such as those of meat, fish meat, fruit and vegetable; alcoholic beverages such as synthetic sake, wine and liquors; soft drinks such as coffee, tea, cocoa, juice, carbonated beverage, sour milk beverage and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix and "sokuseki-shiruco" (an instant mix of adzuki-bean soup with rice cake) and instant soup mix; and beverages such as baby foods, foods for therapy, beverages supplemented with nutrition, peptide foods and frozen foods; as well as for improving the tastes and qualities of the aforementioned food-products.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be also used in feeds and pet foods for animals such as domestic animals, poultry, honey bees, silk worms and fishes in order to improve their taste preferences. These saccharides and **trehalose** can be arbitrarily used as a sweetener, taste-improving agent, quality-improving agent and stabilizer in other products in paste and liquid form such as a tobacco, cigarette, dentifrice, lipstick, rouge, lip cream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachou, oral refrigerant, gargle, cosmetic and pharmaceutical.

DEPR:
The present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be used as a quality-improving agent and stabilizer in biologically active substances which may contain effective ingredients and activities, as well as in health foods and pharmaceuticals containing the biologically active substances. Examples of such a biologically active substance are lymphokines such as .alpha.-, .beta.- and .gamma.-interferons, tumor necrosis factor-.alpha. (TNF-.alpha.), tumor necrosis factor-.beta. (TNF-.beta.), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor and interleukin 2; hormones such as insulin, growth hormone, prolactin, erythropoietin and follicle-stimulating hormone; biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, smallpox vaccine, tetanus toxoid,

Trimeresurus antitoxin and human immunoglobulin; antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin and kanamycin sulfate; vitamins such as thiamine, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol and tocopherol; enzymes such as lipase, elastase, urokinase, protease, .beta.-amylase, isoamylase, glucanase and lactase; extracts such as ginseng extract, snapping turtle extract, chlorella extract, aloe extract and propolis extract; viable microorganisms such as viruses, lactic acid bacteria and yeasts; and other biologically active substances such as royal jelly. By using the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides, the aforementioned biologically active substances are arbitrarily prepared into health foods and pharmaceuticals with a satisfactorily-high stability and quality without a fear of losing or inactivating their effective ingredients and activities.

DEPR:
As described above, the methods for incorporating the present non-reducing saccharides, relatively-low reducing saccharides containing them and/or **trehalose** prepared from these saccharides into the above-mentioned compositions include conventional methods, for example, mixing, kneading, dissolving, melting, soaking, permeating, sprinkling, applying, coating, spraying, injecting, crystallizing and solidifying. These saccharides and **trehalose** are usually incorporated into the above-mentioned compositions in an amount of 0.1% or higher, preferably, one % or higher, d.s.b.

DEPR:
Fifty mg aliquots of non-reducing saccharides P I, P II, P III, P IV and P V in Experiment 4 were respectively dissolved in one ml of 50 mM acetate buffer (pH 4.5), admixed with one unit of glucoamylase commercialized by Seikagaku-Kogyo Co., Ltd., Tokyo, Japan, to effect enzymatic hydrolysis at 40.degree. C. for hours. The only saccharides detected in every resultant mixture on HPLC analysis were glucose and **trehalose**. The contents of the detected glucose and **trehalose**, and their molecular ratios were as shown in Table

DEPR:
As evident from the results in Table 5, it was revealed that (i) the non-reducing saccharide P I was hydrolyzed into one mole of glucose and one mole of **trehalose**; P II, hydrolyzed into two moles of glucose and one mole of **trehalose**; (iii) PIII, hydrolyzed into three moles of glucose and one mole of **trehalose**; (iv) P IV, hydrolyzed into four moles of glucose and one mole of **trehalose**; and (v) P V, hydrolyzed into five moles of glucose and one mole of **trehalose**.

DEPR:
In view of the enzymatic reaction mechanism of glucoamylase, it was revealed that these non-reducing saccharides have a structure of saccharide consisting of one or more moles of glucose bound to one mole of **trehalose** via the .alpha.-1,4 linkage or .alpha.-1,6 linkage: The non-reducing saccharide P I is a non-reducing reducing saccharide having a degree of glucose polymerization of 3 (DP 3) and consisting of one mole of glucose bound to one mole of **trehalose**; P II, a non-reducing saccharide having DP 4 and consisting of two moles of glucose bound to one mole of **trehalose**; P III, a non-reducing saccharide having DP 5 and consisting of three moles of glucose bound to one mole of **trehalose**; P IV, a non-reducing saccharide having DP 6 and consisting of four moles of glucose bound to one mole of **trehalose**; and P V, a non-reducing saccharide having DP 7 and consisting of five moles of glucose bound to one mole of **trehalose**. It was revealed that, when .beta.-amylase was act on these non-reducing saccharides similarly as with glucoamylase, P I and P II were not hydrolyzed but P III, P IV and P V were respectively hydrolyzed into one mole of maltose and one mole of P I, one mole of maltose and one mole of P II, and two moles of maltose and one mole of P I.

DEPR:
Based on these results, it was concluded that the enzymatic reaction of the present non-reducing saccharide-forming enzyme is an intramolecular reaction without changing the molecular weights of the substrates used, i.e. an intramolecular reaction without changing their degrees of glucose polymerization. It was concluded that the non-reducing saccharides P I, P II,

PIII, P IV and P V were the respective .alpha.-glycosyl trehaloses (G.sub.n -T, wherein the symbol "G" means glucose residue; the symbol "n", one or more integers; and the symbol "T", .alpha.,.alpha.-**trehalose** residue) of .alpha.-glucosyl **trehalose,** .alpha.-maltosyl **trehalose,** .alpha.-maltotriosyl **trehalose,** .alpha.-maltotetraosyl **trehalose** and .alpha.-maltopentaosyl **trehalose**.

DEPR:
As evident from the results in Tables 7 and 8, it was revealed that similarly as in Experiment 6 with glucoamylase the saccharide preparations P I, P II, P III, P IV and P V were hydrolyzed by .alpha.-glucosidase and rat intestinal acetone powder into glucose and **trehalose** molecules.

DEPR:
To the resultant hydrolysate obtained with .alpha.-glucosidase or rat intestinal acetone powder was added one unit trehalase derived from pig kidney, an enzyme preparation of Sigma Chemical Company, St., Louis, USA, and the mixture was incubated at pH 5.7 and 37.degree. C. for 18 hours, followed by analyzing the saccharide composition of the resultant mixture on HPLC to reveal that **trehalose,** formed from the saccharide preparations P I, P II, PIII, P IV and P V, was hydrolyzed by trehalase into glucose molecules.

DEPR:
Based on these results, it was concluded that the present non-reducing saccharide-forming enzyme is a novel enzyme which intramolecularly converts a reducing end unit in reducing partial starch hydrolysates to a non-reducing end unit, a **trehalose** residue, i.e. a **trehalose** structure.

DEPR:
By using the purified enzyme preparation obtained in Experiment 10, the preparation and the confirmation of the structure of non-reducing saccharides were conducted in accordance with the methods in Experiments 4 and 6. As a result, it was revealed that the enzyme preparation forms one or more non-reducing saccharides, which saccharide has a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher, when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
In accordance with the method in Experiment 12, non-reducing saccharides were prepared by using partially purified enzyme preparations from these known microorganisms, and their structures were studied to find that, similarly as the non-reducing saccharide-forming enzyme from Rhizobium sp. M-11, every enzyme preparation formed non-reducing saccharides having a **trehalose** structure as an end unit and a degree of glucose polymerization of 3 or higher when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

DEPR:
The following Examples A illustrate the preparation of the present non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose**; and Examples B illustrate compositions containing one or more of these saccharides and **trehalose**.

DEPR:
Forty parts by weight of "PINE-DEX #4", a partial starch hydrolysate commercialized by Matsutani Chemical Ind., Tokyo, Japan, was dissolved in 60 parts by weight of water, and the resultant solution was heated to 45.degree. C., adjusted to pH 6.5, mixed with one unit per g partial starch hydrolysate of a non-reducing saccharide-forming enzyme prepared by the method in Example A-1, and subjected to an enzymatic reaction for 96 hours while keeping at the temperature and pH. Thereafter, the reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme, diluted to give a concentration of about 20%, d.s.b., admixed with 10 units per g partial starch hydrolysate of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and subjected to an enzymatic reaction for 40 hours, followed by heating the resultant mixture to inactivate the remaining enzyme. The mixture thus obtained was in the usual manner decolored with an activated

charcoal, desalted with an ion-exchange resin, and concentrated to give a concentration of about 60%, d.s.b. The saccharide solution thus obtained contained 29.5% **trehalose,** d.s.b. The saccharide solution was column chromatographed in accordance with the method in Example A-2 except that "CG 6000 (Na.sup.+ -form)", a strongly-acidic cation exchange resin commercialized by Japan Organo Co., Ltd., Tokyo, Japan, was used as a resin for fractionation, followed by recovering a **trehalose**-rich fraction. The fraction contained about 90% **trehalose,** d.s.b. The fraction was concentrated into an about 75% solution which was then placed in a crystallizer, admixed with about 2%, d.s.b., hydrous crystalline **trehalose** as a seed crystal and gradually cooled to obtain a massecuite with a degree of crystallization of about 45%. The massecuite was sprayed from a nozzle equipped on the top of a spraying tower at a pressure of 150 kg/cm.sup.2. In the spraying step, the massecuite was simultaneously ventilated with 85.degree. C. hot air sent from the top of the spraying tower, and the resultant crystalline powder was collected on a metal wire netting conveyer provided on the basement of the spraying tower, and gradually moved out of the tower while a stream of 40.degree. C. air was passing upwards through the metal wire netting. The resultant crystalline powder was injected in an ageing tower and aged for 10 hours to complete the crystallization and drying, followed by recovering a powdery hydrous crystalline **trehalose**.

DEPR:
In accordance with the method in Example A-3, 30% suspension of corn starch was subjected to the action of an .alpha.-amylase specimen commercialized by Novo Industri A/S, Copenhagen, Denmark; a maltotetraose forming amylase specimen commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan; and an .alpha.-amylase specimen commercialized by Ueda Chemical Co., Ltd., Osaka, Japan. The resultant mixture was autoclaved at 120.degree. C., cooled to 45.degree. C., admixed with 2 units per g starch of a non-reducing saccharide-forming enzyme prepared by the above-mentioned method, and subjected to an enzymatic reaction for 64 hours. The reaction mixture was heated at 100.degree. C. for 10 min to inactivate the remaining enzyme. In accordance with the method in Example A-6, the resultant solution was subjected to the action of glucoamylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, decolored, desalted and concentrated into an about 60% solution. The saccharide solution thus obtained contained about 25% **trehalose,** d.s.b. The saccharide solution was fractionated on column chromatography using a strongly-acidic cation-exchange resin to obtain fractions rich in **trehalose**. The fractions were pooled, placed in a vessel and boiled down under a reduced pressure into a syrup with a moisture content of about 4.0%. The syrup was placed in a crystallizer and admixed with one % of anhydrous crystalline **trehalose,** as a seed crystal, with respect to the syrup, d.s.b., followed by crystallizing anhydrous crystalline **trehalose** at 95.degree. C. for 5 min while stirring. The resultant was transferred to an aluminum container and aged at 100.degree. C. for 6 hours to form a block. The resultant block was pulverized by a cutting machine and subjected to a fluidized-bed drying to obtain a powdery anhydrous crystalline **trehalose** with a moisture content of about 0.3%.

DEPR:
Three parts by weight of gum base was melted by heating until it softened, and the resultant was mixed with 4 parts by weight of sucrose and 3 parts by weight of a hydrous crystalline **trehalose** powder obtained by the method of Example A-6, and further mixed with adequate amounts of a flavor and a coloring agent. The resultant mixture was kneaded by a roll in the usual manner, formed and packed to obtain the desired product.

DEPR:
Forty % "Hinute S", a peptide solution of edible soy beans commercialized by Fuji Oil Co., Ltd., Tokyo, Japan, was mixed with 2 parts by weight of a powder containing hydrous crystalline **trehalose** prepared by the method of Example A-6, and the resultant mixture was placed in a plastic vessel, dried in vacuo at 50.degree. C., and pulverized to obtain a powdery peptide. The product having a satisfactory taste and flavor can be arbitrarily used as a material for confectioneries such as premixes, sherbets and ice creams, as well as baby foods and therapeutic nutrition in the form of oral and intubation feedings.

DEPR:

Egg yolks prepared from fresh eggs were sterilized at 60.degree.-64.degree. C. by a plate heater, and the resultant liquid was mixed with 4 parts by weight of a powdery anhydrous crystalline **trehalose** prepared by the method of Example A-8 with respect to one part by weight of the liquid. The resultant mixture was transferred to a vessel, allowed to stand overnight to form a block while the anhydrous crystalline **trehalose** was permitted to hydrate to hydrous crystalline **trehalose**. The block thus obtained was pulverized by a cutting machine to obtain a powdery egg yolk.

DEPR:
A crude tablet as a core, 150 mg weight, was coated with a solution consisting of 40 parts by weight of a powdery hydrous crystalline **trehalose** obtained by the method of Example A-6, 2 parts by weight of pullulan having an average molecular weight of 200,000, 30 parts by weight of water, 25 parts by weight of talc, and 3 parts by weight of titanium oxide until the total weight reached to about 230 mg, and the resultant was further coated with a solution consisting of 65 parts by weight of a fresh preparation of the same powdery hydrous crystalline **trehalose**, one part by weight of pullulan, and 34 parts by weight of water, and glossed with a liquid wax to obtain a sugar coated tablet having a satisfiable gloss and appearance.

DEPR:
As evident from above, the present novel non-reducing saccharide-forming enzyme converts reducing partial starch hydrolysates into non-reducing saccharides in a satisfactorily-high yield under a relatively-mild enzymatic reaction condition without changing the degrees of glucose polymerization of the reducing partial starch hydrolysates. The non-reducing saccharides, which can be readily separated and purified, and relatively-low reducing saccharides containing them, as well as **trehalose** prepared from these saccharides, have a satisfactory stability, quality and mild sweetness. These products are assimilated and utilized as an energy source by the body when orally administered. These non-reducing saccharides, relatively-low reducing saccharides containing them, and **trehalose** prepared from these saccharides can be arbitrarily used in compositions such as food products, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer and filler.

DEPR:
Thus, the present invention provides a novel technique to prepare in an industrial-scale and at a relatively-low cost non-reducing saccharides, which could not have been readily obtained in spite of their great demands, by using reducing partial starch hydrolysates prepared from starch as a cheap and abundant source, as well as to prepare relatively-low reducing saccharides containing the non-reducing saccharides, and **trehalose** prepared from these saccharides. The present invention has a great influence on the fields such as starch-, enzyme- and biochemical-sciences; and other industrial fields, especially, food-, cosmetic- and pharmaceutical-industries, as well as forestry, fisheries, and agricultural-, livestock- and chemical-industries. Thus, the influence of the present invention on these fields is unfathomable.

DEPV:
(1) The present non-reducing saccharide-forming enzyme forms non-reducing saccharides having a **trehalose** structure when allowed to act on one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher without changing their degrees of glucose polymerization; and

DEPV:
(2) The non-reducing saccharide P V is mainly hydrolyzed by .alpha.-amylase into the non-reducing saccharide P II and maltotriose, while the non-reducing saccharide P II is hydrolyzed by glucoamylase into one mole of **trehalose** and two moles of glucose.

DETL:
Carbon source Utilization Acid formation D-Glucose + + D-Galactose + + D-Fructose + + L-Arabinose + + D-Xylose + + L-Rhamnose + + Maltose + - Sucrose + + Lactose + - **Trehalose** + - Raffinose + + Mannitol + - Dextrin + - Dulcitol + -

DETL:
TABLE 5 ______________________________ Saccharide Glucose **Trehalose** Molecular ratio preparation (%) (%) (Glucose/Trehalose) ______________________________ P I 36.2 63.8 1.07 P II 52.0 48.0 2.06 P III 61.4 38.6 3.02 P IV 68.3 31.7 4.09 P V 72.9 27.1 5.11 ______________________________

DETL:
TABLE 7 ______________________________ Saccharide composition of hydrolysate with .alpha.-glucosidase Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________ P I 36.5 63.0 0.5 P II 52.1 47.6 0.3 P III 61.7 38.1 0.2 P IV 69.5 30.2 0.3 P V 71.4 28.3 0.3 ______________________________

DETL:
TABLE 8 ______________________________ Saccharide composition of hydrolysate with rat intestinal acetone powder Glucose **Trehalose** Other saccharides Saccharide (%) (%) (%) ______________________________ P I 37.2 62.4 0.4 P II 52.5 47.1 0.4 P III 62.0 37.6 0.4 P IV 68.8 30.8 0.4 P V 73.4 26.5 0.1 ______________________________

CLPR:
1. An isolated enzyme which converts a reducing partial starch hydrolysate to a non-reducing saccharide having a **trehalose** structure, said non-reducing saccharide comprising one mole of **trehalose** and at east one mole of glucose.

CLPR:
2. The isolated enzyme of claim 1, wherein the **trehalose** structure in said non-reducing saccharide is located in its end unit, and said reducing partial starch hydrolysate is one or more reducing partial starch hydrolysates having a degree of glucose polymerization of 3 or higher.

ORPL:
Database WPI, Section Ch, Week 9425, Derwent Publications Ltd., London, GB; Class D16, AN 94-206435, "Alpha, alpha-**trehalose** production comprising extraction from yeast and water, and treatment with ultrafiltration or reverse osmosis", & JP,A,06 145 186 (Kirin Brewery KK & Nippon Shokuhin Kako KK), May 24, 1994, *abstract*.

ORPL:
Hoelzle et al., "Increased Accumulation of **Trehalose** in Rhizobia Cultured under 1% Oxygen," Applied and Environmental Microbiology, pp. 3213-3215, Oct. 1990.

ORPL:
Journal of the Chemical Society, May 1965 Letchworth, GB, pp. 3489-3490, Birch, "A method of obtaining crystalline anhydrous alphaalpha-**trehalose**".

ORPL:
Biotechnology Letters, vol. 12, No. 6, Jun. 1990, pp. 431-432; Lama et al; "Starch conversion with immobilized thermophilic Archaebacterium **sulfolobus** solfataricus".

USPT

US-CL-CURRENT: 435/10,435/16 ,435/174 ,435/175 ,435/178 ,435/25 ,436/817 ,536/1.11 ,536/26.24 ,536/4.1 ,568/347

US-PAT-NO: 5356790
DOCUMENT-IDENTIFIER: US 5356790 A
TITLE: Highly sensitive assay method for myo-inositol, composition for practicing same, novel myo-inositol dehydrogenase, and process for producing same
DATE-ISSUED: October 18, 1994
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Ueda; Shigeru	Shizuoka	N/A	N/A	JPX
Takahashi; Mamoru	Shizuoka	N/A	N/A	JPX
Misaki; Hideo	Shizuoka	N/A	N/A	JPX
Imamura; Shigeyuki	Shizuoka	N/A	N/A	JPX
Matsuura; Kazuo	Shizuoka	N/A	N/A	JPX

US-CL-CURRENT: 435/26,435/10 ,435/16 ,435/174 ,435/175 ,435/178 ,435/25 ,436/817 ,536/1.11 ,536/26.24 ,536/4.1 ,568/347
ABSTRACT:
Myo-inositol in a specimen is assayed by reacting a specimen containing myo-inositol with:

a) myo-inositol dehydrogenase using a thio-NADP group or thio-NAD group and an NADP group or NAD group as coenzymes, and which catalyzes a reversible reaction forming myo-inosose from myo-inositol,

b) A.sub.1 and

c) B.sub.1

to effect a cycling reaction ##STR1## wherein A.sub.1 is a thio-NADP group, thio-NAD group, NADP group or NAD group, A.sub.2 is a reduced form of A.sub.1, when A.sub.1 is a thio-NADP group or thio-NAD group, B.sub.1 is a reduced NADP group or reduced NAD group and when A.sub.1 is an NADP group or NAD group, B.sub.1 is a reduced thio-NADP group or reduced thio-NAD group, and wherein B.sub.2 is an oxidized form of B.sub.1. The change in the amount of A.sub.2 generated or B.sub.1 consumed by the cycling reaction is measured to perform the assay. A composition for performing the assay comprises the above myo-inositol dehydrogenase, as well as the above components A.sub.1 and B.sub.1. The myo-inositol dehydrogenase can be produced by culturing a suitable microorganism belonging to genus Bacillus, particularly Bacillus sp. No. 3 FERM BP-3013.
5 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

DEPR:
Bacillus **acidocaldarius**, B. subtilis, B. badius, B. brevis, B. coagulans, B. licheniformis, B. pantothenticus, B. schegelli and B. stearothermophilus.

DETL:
____ Gram-strain + KOH reaction - Capsule formation - Acid fastness stain - OF-test (High Leifson) NT OF-test (nitrogen source: NH.sub.4 H.sub.2 PO.sub.4) F Aerobic growth + Anaerobic growth + Growth temperature 70.degree. C. - 60.degree. C. + 37.degree. C. + 30.degree. C. - Halotolerant NaCl conc. (%) 0% + 3% + 5% - Growth pH pH 5.6 - pH 6.2 + pH 9.0 + Gelatin hydrolysis - Starch hydrolysis (+) Casein hydrolysis - Esculin hydrolysis + Cellulose hydrolysis - Tyrosine hydrolysis - Arginine hydrolysis - Catalase production + Oxidase production + Lecithinase production - Urease production (SSR) - Urease production (Chris) - Indol production - H.sub.2 S production - (detection: lead acetate paper) Acetoin production (K.sub.2 HPO.sub.4) - Acetoin production (NaCl) - MR test - Nitrate reduction Gas detection - NO.sub.2 -- - NO.sub.3 -- + Utilization of Simmons medium Citrate - Malate - Maleate - Malonate -

Propionate - Gluconate - Succinate - Utilization of Christenseen medium Citrate + Malate - Maleate - Malonate - Propionate + Gluconate - Succinate - Gas production from glucose - Acid formation from sugar Adonitol - L (+) arabinose - Cellobiose + Dulsitol - Meso-erythritol - Fructose + Fucose + Galactose + Glucose + Glycerin + Inositol + Inulin + Lactose + Maltose + Mannitol + Mannose + Melezitose - Melibiose + Raffinose - Rhamnose + D-ribose + Salicin + L-sorbose - Sorbitol - Starch + Saccharose + Xylose - **Trehalose** + ________________________________ (+ = positive, (+) = weakly positive, - = negative, NT = not tested)

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	241	sulfolobus or acidocaldarius	USPAT	2000/10/19 13:50
2	L2	2860	trehalose	USPAT	2000/10/19 13:50
3	L3	23	1 and 2	USPAT	2000/10/19 14:56
4	L6	86	non adj reducing adj saccharide$1	USPAT	2000/10/19 14:57
5	L7	15	1 and 6	USPAT	2000/10/19 14:57

	Document ID	Issue Date	Pages	Title
1	US 6129788 A	20001010	19	Method of producing saccharide preparations
2	US 6027918 A	20000222	28	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
3	US 6017899 A	20000125	30	Non-reducing saccharide-forming enzyme, its preparation and uses
4	US 5976856 A	19991102	27	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
5	US 5922578 A	19990713	28	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
6	US 5919668 A	19990706	40	Non-reducing saccharide and its production and use
7	US 5863771 A	19990126	13	Saccharide composition comprising maltooligosylturanose and maltooligosylpalatinose, its preparation and uses
8	US 5856146 A	19990105	27	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
9	US 5789392 A	19980804	40	Saccharide composition with reduced reducibility, and preparation and uses thereof
10	US 5723327 A	19980303	22	Thermostable trehalose-releasing enzyme, and its preparation and uses

	Document ID	Issue Date	Pages	Title
11	US 5716838 A	19980210	30	Non-reducing saccharide-forming enzyme, its preparation and uses
12	US 5714368 A	19980203	19	Thermostable non-reducing saccharide-forming enzyme its production and uses
13	US 5681826 A	19971028	41	Saccharide composition with reduced reducibility, and preparation and uses thereof
14	US 5677442 A	19971014	30	Method of crystallizing trehalose without using organic solvent
15	US 5610047 A	19970311	29	Non-reducing saccharide-forming enzyme, its preparation and uses

USPT

US-CL-CURRENT: 435/105,435/96 ,435/98

US-PAT-NO: 6129788
DOCUMENT-IDENTIFIER: US 6129788 A
TITLE: Method of producing saccharide preparations
DATE-ISSUED: October 10, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Liaw; Gin C.	Decatur	IL	N/A	N/A
Pedersen; Sven	Gentofte	N/A	N/A	DKX
Hendriksen; Hanne Vang	Holte	N/A	N/A	DKX
Svendsen; Allan	Birker.o	N/A	N/A	DKX
Nielsen; Bjarne R.o	slashed.d	N/A	N/A	DKX
slashed.nfeldt	Virum	N/A	N/A	DKX
Nielsen; Ruby Illum	Farum			

US-CL-CURRENT: 127/40,435/105 ,435/96 ,435/98

ABSTRACT:
The present invention relates to a method for the production of saccharide preparations, i.e., syrups, by saccharifying a liquefied starch solution, which method comprises a saccharification step during which step one or more enzymatic saccharification stages takes place, and the subsequent steps of one or more high temperature membrane separation steps, and recirculation of the saccharification enzyme, in which method the membrane separation steps are carried out as an integral part of the saccharification step.

16 Claims, 5 Drawing figures
Exemplary Claim Number: 1,9
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 435/183,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ,536/23.2 ,536/23.7

US-PAT-NO: 6027918
DOCUMENT-IDENTIFIER: US 6027918 A
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: February 22, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/69.2,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ;536/23.2 ,536/23.7
ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of trehalose on an industrial scale and in a satisfactorily-high yield.
13 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 426/658,435/100 ,514/54 ,514/61 ,514/777 ,514/778

US-PAT-NO: 6017899
DOCUMENT-IDENTIFIER: US 6017899 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: January 25, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/53,426/658 ,435/100 ,514/54 ,514/61 ,514/777 ,514/778

ABSTRACT:

Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.

14 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/101,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

US-PAT-NO: 5976856
DOCUMENT-IDENTIFIER: US 5976856 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: November 2, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/201,435/101 ,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

1 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 435/101,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

US-PAT-NO: 5922578
DOCUMENT-IDENTIFIER: US 5922578 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: July 13, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/101 ,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.
7 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 435/100,435/101 ,435/72 ,435/74 ,435/95 ,435/96 ,435/98 ,435/99 ,536/123.1 ,536/123.13

US-PAT-NO: 5919668
DOCUMENT-IDENTIFIER: US 5919668 A
TITLE: Non-reducing saccharide and its production and use
DATE-ISSUED: July 6, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mandai; Takahiko	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/100 ,435/101 ,435/72 ,435/74 ,435/95 ,435/96 ,435/98 ,435/99 ,536/123.1 ,536/123.13
ABSTRACT:
In the production of non-reducing saccharides such as trehalose, alpha-glycosyl trehaloses and alpha-glycosyl alpha-glycosides where a solution of liquefied starch is subjected either to non-reducing saccharide-forming enzyme or non-reducing saccharide-forming enzyme and trehalose-releasing enzyme, combinations with starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase improve the yields for such non-reducing saccharides to levels which are hardly attainable only with reducing-saccharide-forming enzyme and trehalose-releasing enzyme. The non-reducing saccharides and less reducing reducing saccharides containing the same commonly bear a variety of desirable properties which make them useful in a variety of compositions including food products, cosmetics and medicines.
22 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

USPT

US-CL-CURRENT: 426/658,435/100 ,435/72 ,435/99 ,514/54 ,514/61 ,514/777 ,536/1.11 ,536/123.1 ,536/124 ,536/4.1

US-PAT-NO: 5863771
DOCUMENT-IDENTIFIER: US 5863771 A
TITLE: Saccharide composition comprising maltooligosylturanose and maltooligosylpalatinose, its preparation and uses
DATE-ISSUED: January 26, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Aga; Hajime	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Fukuda; Shigeharu	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/101,426/658 ,435/100 ,435/72 ,435/99 ,514/54 ,514/61 ,514/777 ,536/1.11 ,536/123.1 ,536/124 ,536/4.1
ABSTRACT:
A saccharide composition comprising maltooligosyl derivatives of turanose and palatinose which can be readily produced, separated, and purified in a relatively-high yield from maltooligosylsucrose by allowing non-reducing saccharide-forming enzymes to act on aqueous solutions containing maltooligosylsucrose. These saccharides are reducing oligosaccharides with a mild and high-quality sweetness and can be used orally and parenterally, as well as being readily metabolized and used by living bodies.
17 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

USPT

US-CL-CURRENT: 435/100,435/195 ,435/200 ,435/201 ,435/253.3 ,435/822 ,530/350 ,530/825

US-PAT-NO: 5856146
DOCUMENT-IDENTIFIER: US 5856146 A
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: January 5, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/100 ,435/195 ,435/200 ,435/201 ,435/253.3 ,435/822 ,530/350 ,530/825
ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of trehalose on an industial scale and in a satisfactorily-high yield.
6 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 536/123.1,536/124

US-PAT-NO: 5789392
DOCUMENT-IDENTIFIER: US 5789392 A
TITLE: Saccharide composition with reduced reducibility, and preparation and uses thereof
DATE-ISSUED: August 4, 1998
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/54,536/123.1 ,536/124
ABSTRACT:
A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of trehalose and/or saccharides having a trehalose structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction.
9 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

USPT

US-CL-CURRENT: 435/100,435/193 ,435/195 ,435/200 ,536/123.13

US-PAT-NO: 5723327
DOCUMENT-IDENTIFIER: US 5723327 A
TITLE: Thermostable trehalose-releasing enzyme, and its preparation and uses
DATE-ISSUED: March 3, 1998
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Ikegami; Shouji	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/201,435/100 ,435/193 ,435/195 ,435/200 ,536/123.13
ABSTRACT:
Disclosed are novel thermostable trehalose-releasing enzyme, and its preparations and uses. The enzyme is obtainable from the culture of microorganisms such as Sulfolobus acidocaldarius (ATCC 33909 and ATCC 49426) and Sulfolobus solfataricus (ATCC 35091 and ATCC 35092), and capable of hydrolyzing at a temperature of over 55.degree. C. the linkage between a trehalose moiety and the remaining glycosyl moiety in a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. Trehalose and compositions containing the same are extensively useful in food products, cosmetics and pharmaceuticals.
15 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 435/100,435/101 ,435/252.1 ,536/123.1 ,536/123.13

US-PAT-NO: 5716838
DOCUMENT-IDENTIFIER: US 5716838 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: February 10, 1998
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/252.2,435/100 ,435/101 ,435/252.1 ,536/123.1 ,536/123.13
ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act-on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.
12 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/100,435/101 ,435/200 ,514/53 ,514/54 ,514/60 ,536/123.13 ,536/124

US-PAT-NO: 5714368
DOCUMENT-IDENTIFIER: US 5714368 A
TITLE: Thermostable non-reducing saccharide-forming enzyme its production and uses
DATE-ISSUED: February 3, 1998
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nakada; Tetsuya	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/201,435/100 ,435/101 ,435/200 ,514/53 ,514/54 ,514/60 ,536/123.13 ,536/124

ABSTRACT:
Disclosed are novel thermostable non-reducing saccharides-forming enzyme, its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Sulfolobus acidocaldarius (ATCC 33909 and ATCC 49426) and Sulfolobus solfataricus (ATCC 35091 and ATCC 35092), and capable of forming non-reducing saccharides having a trehalose structure as an end unit when allowed to act on reducing partial starch hydrolysates at a temperature of over 55.degree. C. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.
23 Claims, 4 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 4

USPT

US-CL-CURRENT: 536/123.1

US-PAT-NO: 5681826
DOCUMENT-IDENTIFIER: US 5681826 A
TITLE: Saccharide composition with reduced reducibility, and preparation and uses thereof
DATE-ISSUED: October 28, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/54,536/123.1

ABSTRACT:
A saccharide composition with a reduced reducibility which is prepared by hydrogenating a saccharide mixture comprising reducing saccharides and non-reducing saccharides consisting of trehalose and/or saccharides having a trehalose structure. The saccharide composition has a satisfactory sweetness, taste and stability, and is substantially free from reducibility, so that it can be freely used in a variety of compositions such as foods, cosmetics and pharmaceuticals which are susceptible to reduction.
19 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

USPT

US-CL-CURRENT: 435/100,536/102 ,536/123.1 ,536/127

US-PAT-NO: 5677442
DOCUMENT-IDENTIFIER: US 5677442 A
TITLE: Method of crystallizing trehalose without using organic solvent
DATE-ISSUED: October 14, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 536/123.13,435/100 ,536/102 ,536/123.1 ,536/127
ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals. Also disclosed is a method of crystallizing trehalose from a 65 to 90% aqueous solution in the absence of organic solvent.
3 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/200,435/95 ,435/96 ,435/97 ,435/99

US-PAT-NO: 5610047
DOCUMENT-IDENTIFIER: US 5610047 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: March 11, 1997
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/201,435/200 ,435/95 ,435/96 ,435/97 ,435/99
ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.
2 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	241	sulfolobus or acidocaldarius	USPAT	2000/10/19 13:50
2	L2	2860	trehalose	USPAT	2000/10/19 15:13
3	L3	23	1 and 2	USPAT	2000/10/19 14:56
4	L6	86	non adj reducing adj saccharide$1	USPAT	2000/10/19 14:57
5	L7	15	1 and 6	USPAT	2000/10/19 14:57
6	L8	2879	$trehalose	USPAT	2000/10/19 15:13
7	L9	23	1 and 8	USPAT	2000/10/19 15:14
8	L10	83	(2 or 4) near6 (synthes$8 or produc$8 or form$6) near6 enzym$8	USPAT	2000/10/19 15:21

	Document ID	Issue Date	Pages	Title
1	US 6133034 A	20001017	29	Methods and compositions related to the production of trehalose
2	US 6130368 A	20001010	21	Transgenic plants producing trehalose
3	US 6129788 A	20001010	19	Method of producing saccharide preparations
4	US 6126962 A	20001003	13	Crystalline powdery saccharide, its preparation and uses
5	US 6110707 A	20000829	136	Recombinant expression of proteins from secretory cell lines
6	US 6090792 A	20000718	38	Maltose-trehalose converting enzyme, and preparation and uses thereof
7	US 6087129 A	20000711	79	Recombinant expression of proteins from secretory cell lines
8	US 6087146 A	20000711	36	Recombinant thermostable enzyme for converting maltose into trehalose
9	US 6027918 A	20000222	28	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
10	US 6017899 A	20000125	30	Non-reducing saccharide-forming enzyme, its preparation and uses
11	US 6013488 A	20000111	8	Method for reverse transcription

	Document ID	Issue Date	Pages	Title
12	US 5993889 A	19991130	28	Trehalose phosphorylase, its preparation and use
13	US 5981498 A	19991109	9	Agent for improving the blood circulation
14	US 5976856 A	19991102	27	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
15	US 5965411 A	19991012	38	Maltose-trehalose converting enzyme, and preparation and uses thereof
16	US 5952204 A	19990914	17	.beta.-fructofuranosidase, its preparation and uses
17	US 5939308 A	19990817	17	Heat resistant maltose phosphorylase, process for preparation thereof, bacteria used for preparation thereof, and methods for using the enzyme
18	US 5935827 A	19990810	16	Maltose phosphorylase, trehalose phosphorylase, novel strain of genus plesiomonas capable of producing these enzymes and process for producing trehalose

	Document ID	Issue Date	Pages	Title
19	US 5935636 A	19990810	32	Trehalose and its production and use
20	US 5925804 A	19990720	30	Production of trehalose in plants
21	US 5922691 A	19990713	21	Crystalline maltotetraosyl glucoside, and its production and use
22	US 5922578 A	19990713	28	Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
23	US 5922580 A	19990713	30	Non-reducing saccharide-forming enzyme, its preparation and uses
24	US 5919668 A	19990706	40	Non-reducing saccharide and its production and use
25	US 5916371 A	19990629	13	Crystalline powdery saccsharide, its preparation and uses

	Document ID	Issue Date	Pages	Title
26	US 5916881 A	19990629	13	High trehalose content syrup
27	US 5912361 A	19990615	5	Process for producing D-glucuronolactone
28	US 5912330 A	19990615	23	Crystalline maltosyl glucoside, and its production and use
29	US 5910436 A	19990608	28	Trehalose phosphorylase, its preparation and uses
30	US 5908767 A	19990601	42	Non-reducing saccharide and its production and use
31	US 5906924 A	19990525	7	Process for producing trehalose derivatives
32	US 5892026 A	19990406	11	High Trehalose content syrup
33	US 5891717 A	19990406	78	Methods and compositions for inhibiting hexokinase
34	US 5883243 A	19990316	18	Non-reducing saccharides, their preparations and uses
35	US 5876975 A	19990302	29	Trehalose phosphorylase its preparation and uses

	Document ID	Issue Date	Pages	Title
36	US 5871993 A	19990216	42	DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparations and uses
37	US 5871994 A	19990216	41	DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparation and uses
38	US 5871977 A	19990216	42	DNA encoding enzyme recombinant DNA and enzyme transformant, and their preparation and uses
39	US 5858992 A	19990112	18	Non-reducing saccharides, their preparation and use
40	US 5858735 A	19990112	4	Process for producing trehalose
41	US 5856146 A	19990105	27	Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
42	US 5854067 A	19981229	77	Hexokinase inhibitors
43	US 5843748 A	19981201	28	Trehalose phosphorylase its preparation and uses
44	US 5837527 A	19981117	17	.beta.-fructofuranosidase its preparation and uses
45	US 5834287 A	19981110	40	DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparation and uses
46	US 5830715 A	19981103	39	DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparations and uses
47	US 5827715 A	19981027	17	Heat resistant maltose phosphorylase, process for preparation thereof, bacteria used for preparation thereof, and methods for using the enzyme
48	US 5824521 A	19981020	11	Saccharide composition containing trehalulose, its preparation and uses

	Document ID	Issue Date	Pages	Title
49	US 5807719 A	19980915	16	Maltose phosohorylase, trehalose phosphorylase, Plesiomonas strain and preparation process of trehalose
50	US 5792921 A	19980811	91	Increasing the trehalose content of organisms by transforming them with combinations of the structural genes for trehalose synthase
51	US 5789392 A	19980804	40	Saccharide composition with reduced reducibility, and preparation and uses thereof
52	US 5780620 A	19980714		Non-reducing oligosaccharides and their production and use
53	US 5773282 A	19980630		Recombinant thermostable enzyme for converting maltose into trehalose from Thermus aquaticus
54	US 5763228 A	19980609		Recombinant enzyme for converting maltose into trehalose from pimelobacter sp.
55	US 5759610 A	19980602		Trehalose and its production and use
56	US 5753469 A	19980519		.beta.-fructofuranosidase, its preparation and uses
57	US 5750389 A	19980512		Purified saccharose-synthase, process for its production and its use

	Document ID	Issue Date	Pages	Title
58	US 5747300 A	19980505		Trehalose and its production and use
59	US 5736380 A	19980407		Maltose-trehalose converting enzyme, and preparation and uses thereof
60	US 5723327 A	19980303		Thermostable trehalose-releasing enzyme, and its preparation and uses
61	US 5716838 A	19980210		Non-reducing saccharide-forming enzyme, its preparation and uses
62	US 5716813 A	19980210		DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparations and uses
63	US 5714368 A	19980203		Thermostable non-reducing saccharide-forming enzyme its production and uses
64	US 5705378 A	19980106		Maltose phosphorylase, trehalose phosphorylase, novel strain of genus plesiomonas capable of producing these enzymes and process for producing trehalose
65	US 5681826 A	19971028		Saccharide composition with reduced reducibility, and preparation and uses thereof
66	US 5677442 A	19971014		Method of crystallizing trehalose without using organic solvent
67	US 5643775 A	19970701		Trehalose phosphorylase and preparation thereof
68	US 5610047 A	19970311		Non-reducing saccharide-forming enzyme, its preparation and uses
69	US 5593869 A	19970114		Method of manufacturing sugars by trehalase
70	US 5591611 A	19970107		Trehalose-releasing enzyme, and its preparation and uses
71	US 5591612 A	19970107		Trehalose-releasing enzyme, and its preparation and uses
72	US 5587290 A	19961224		Stress tolerant yeast mutants

	Document ID	Issue Date	Pages	Title
73	US 5576303 A	19961119		Energy-supplementing saccharide source and its uses
74	US 5565341 A	19961015		Process for producing trehalose
75	US 5556781 A	19960917		DNA encoding enzyme, recombinant DNA and enzyme, transformant, and their preparations and uses
76	US 5538883 A	19960723		Maltose-trehalose converting enzyme
77	US 5529927 A	19960625		"Alga species lobsphaera TM-33 (ATCC 75630) which is useful for preparing trehalase"
78	US 5472863 A	19951205		Trehalose-releasing enzyme
79	US 5455168 A	19951003		.alpha.-glycosyl trehalose-forming enzyme
80	US 5441644 A	19950815		Method of isolation and purification of trehalose
81	US 5422254 A	19950606		Method to increase the trehalose content of organisms by transforming them with the structural genes for the short and long chains of yeast trehalose synthase
82	US 5312909 A	19940517		Recombinant DNA encoding neutral trehalase
83	US 5169767 A	19921208		Method of producing trehalose

USPT

US-CL-CURRENT: 435/183,435/320.1 ,435/410 ,435/69.1 ,435/70.1 ,435/71.1 ,536/23.2 ,536/23.7 ,536/24.1

US-PAT-NO: 6133034
DOCUMENT-IDENTIFIER: US 6133034 A
TITLE: Methods and compositions related to the production of trehalose
DATE-ISSUED: October 17, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Strom; Arne Reidar	Tromso	N/A	N/A	NOX
Kaasen; Inga	Tromso	N/A	N/A	NOX
Styrvold; Olaf Bay	Tromso	N/A	N/A	NOX
McDougall; John	Tromso	N/A	N/A	NOX

US-CL-CURRENT: 435/419,435/183 ,435/320.1 ,435/410 ,435/69.1 ,435/70.1 ,435/71.1 ,536/23.2 ,536/23.7 ,536/24.1

ABSTRACT:
This invention relates to genes involved in the biosynthesis of trehalose. The genes encode trehalose-6-phosphate synthase (trehalose synthase) and trehalose-6-phosphate phosphatase (trehalose phosphatase).

19 Claims, 13 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 13

USPT

US-CL-CURRENT: 800/278,800/284 ,800/287 ,800/288

US-PAT-NO: 6130368
DOCUMENT-IDENTIFIER: US 6130368 A
TITLE: Transgenic plants producing trehalose
DATE-ISSUED: October 10, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Londesborough; John	Helsinki	N/A	N/A	FIX
Tunnela; Outi	Espoo	N/A	N/A	FIX
Holmstrom; Kjell-Ove	Uppsala	N/A	N/A	SEX
Mantyla ; Einar	Uppsala	N/A	N/A	SEX
Welin; Bjorn	Uppsala	N/A	N/A	SEX
Mandal; Abul	Uppsala	N/A	N/A	SEX
Palva; Tapio E.	Porvoo	N/A	N/A	FIX

US-CL-CURRENT: 800/298,800/278 ,800/284 ,800/287 ,800/288
ABSTRACT:
The present invention concerns transgenic plants producing trehalose and methods of increasing the trehalose content of plants. According to the invention, the plants of interest are transformed with the coding sequence of a gene for trehalose-6-phosphate synthase fused to a non-constitutive plant promoter, which allows for temporal, topological or stress-induced control over the expression of the gene. The invention can be used for protecting staple crop plants against drought, high salinity or temperature extremes and for improving the storage properties of harvested plants including green food stuffs, picked fruits and ornamental plants.
22 Claims, 15 Drawing figures
Exemplary Claim Number: 15
Number of Drawing Sheets: 7

USPT

US-CL-CURRENT: 435/105,435/96 ,435/98

US-PAT-NO: 6129788
DOCUMENT-IDENTIFIER: US 6129788 A
TITLE: Method of producing saccharide preparations
DATE-ISSUED: October 10, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Liaw; Gin C.	Decatur	IL	N/A	N/A
Pedersen; Sven	Gentofte	N/A	N/A	DKX
Hendriksen; Hanne Vang	Holte	N/A	N/A	DKX
Svendsen; Allan	Birker.o	N/A	N/A	DKX
Nielsen; Bjarne R.o	slashed.d	N/A	N/A	DKX
slashed.nfeldt	Virum	N/A	N/A	DKX
Nielsen; Ruby Illum	Farum			

US-CL-CURRENT: 127/40,435/105 ,435/96 ,435/98
ABSTRACT:
The present invention relates to a method for the production of saccharide preparations, i.e., syrups, by saccharifying a liquefied starch solution, which method comprises a saccharification step during which step one or more enzymatic saccharification stages takes place, and the subsequent steps of one or more high temperature membrane separation steps, and recirculation of the saccharification enzyme, in which method the membrane separation steps are carried out as an integral part of the saccharification step.
16 Claims, 5 Drawing figures
Exemplary Claim Number: 1,9
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 127/29,424/409 ,424/439 ,424/465 ,424/479 ,424/49 ,514/777 ,514/970

US-PAT-NO: 6126962
DOCUMENT-IDENTIFIER: US 6126962 A
TITLE: Crystalline powdery saccharide, its preparation and uses
DATE-ISSUED: October 3, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Mukai; Kazuhisa	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 424/442,127/29 ,424/409 ,424/439 ,424/465 ,424/479 ,424/49 ,514/777 ,514/970

ABSTRACT:
A stable crystalline powdery saccharide having a crystallinity of 40% or more, less hygroscopicity, satisfactory fluidity, and beneficial handleability, which is obtainable from an aqueous solution, containing trehalose and a different saccharide(s) crystallizable in the presence of trehalose, by crystallizing the trehalose along with the different saccharide(s).

17 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 3

USPT

US-CL-CURRENT: 435/69.1

US-PAT-NO: 6110707
DOCUMENT-IDENTIFIER: US 6110707 A
TITLE: Recombinant expression of proteins from secretory cell lines
DATE-ISSUED: August 29, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Newgard; Christopher B.	Dallas	TX	N/A	N/A
Halban; Philippe	Geneva	N/A	N/A	CHX
Normington; Karl D.	Dallas	TX	N/A	N/A
Clark; Samuel A.	Rockwall	TX	N/A	N/A
Thigpen; Anice E.	Dallas	TX	N/A	N/A
Quaade; Christian	Dallas	TX	N/A	N/A
Kruse; Fred	Dallas	TX	N/A	N/A
McGarry; Dennis	Dallas	TX	N/A	N/A

US-CL-CURRENT: 435/69.4,435/69.1
ABSTRACT:
The present invention a provides methods for production of heterologous polypeptides, for example amylin, using recombinantly engineered cell lines. Also described are methods engineering cells for high level expression, methods of large scale heterologous protein production, methods for treatment of disease in vivo using viral delivery systems and recombinant cell lines, and methods for isolating novel amylin receptors.
41 Claims, 39 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 31

USPT

US-CL-CURRENT: 435/100,435/105 ,514/23 ,536/123.1 ,536/123.13

US-PAT-NO: 6090792
DOCUMENT-IDENTIFIER: US 6090792 A
TITLE: Maltose-trehalose converting enzyme, and preparation and uses thereof
DATE-ISSUED: July 18, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nishimoto; Tomoyuki	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/53,435/100 ,435/105 ,514/23 ,536/123.1 ,536/123.13

ABSTRACT:
An enzyme, which has a molecular weight of about 57,000-120,000 daltons on SDS-PAGE and a pI of about 3.8-5.1 on isoelectrophoresis using ampholyte, converts maltose into trehalose and vice versa. The enzyme was isolated from microorganisms of the genera Pimelobacter, Pseudomonas and Thermus. By using the **enzyme, trehalose is readily formed** from a commercially available maltose in an industrial scale and a relatively-low cost. Trehalose and saccharide compositions containing the same, which are preparable with the enzyme, are suitably used in food products, cosmetic compositions and pharmaceutical compositions.
5 Claims, 12 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 12

USPT

US-CL-CURRENT: 435/6,435/69.1

US-PAT-NO: 6087129
DOCUMENT-IDENTIFIER: US 6087129 A
TITLE: Recombinant expression of proteins from secretory cell lines
DATE-ISSUED: July 11, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Newgard; Christopher B.	Dallas	TX	N/A	N/A
Normington; Karl D.	Dallas	TX	N/A	N/A
Clark; Samuel A.	Rockwall	TX	N/A	N/A
Thigpen; Anice E.	Dallas	TX	N/A	N/A
Quaade; Christian	Dallas	TX	N/A	N/A
Kruse; Fred	Dallas	TX	N/A	N/A

US-CL-CURRENT: 435/69.4,435/6 ,435/69.1

ABSTRACT:
The present invention a provides methods for production of heterologous polypeptides using a variety recombinantly engineered secretory cell lines. The common feature of these cell lines is the absence of expression of at least one endogenous polypeptide. The host cell machinery normally used to produce the endogenous polypeptide is then usurped for the purpose of making the heterologous polypeptide. Also described are methods engineering cells for high level expression, methods of large scale protein production, and methods for treatment of disease in vivo using viral delivery systems and recombinant cell lines.

26 Claims, 16 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

USPT

US-CL-CURRENT: 435/100,435/252.3 ,435/440 ,536/23.2 ,536/24.3

US-PAT-NO: 6087146
DOCUMENT-IDENTIFIER: US 6087146 A
TITLE: Recombinant thermostable enzyme for converting maltose into trehalose
DATE-ISSUED: July 11, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tsusaki; Keiji	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/200,435/100 ,435/252.3 ,435/440 ,536/23.2 ,536/24.3
ABSTRACT:
Disclosed are a recombinant thermostable enzyme, which converts maltose into trehalose and is stable up to a temperature of about 80.degree. C. even when incubated at pH 7.0 for 60 min, a preparation of the enzyme, a DNA encoding the enzyme, a recombinant DNA containing the DNA, a transformant, and an enzymatic conversion method of maltose by using the enzyme.
5 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 435/183,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ,536/23.2 ,536/23.7

US-PAT-NO: 6027918
DOCUMENT-IDENTIFIER: US 6027918 A
TITLE: Recombinant thermostable enzyme which releases trehalose from non-reducing saccharide
DATE-ISSUED: February 22, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mitsuzumi; Hitoshi	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/69.2,435/183 ,435/200 ,435/252.33 ,435/320.1 ,435/69.1 ,435/71.1 ,435/71.2 ,536/23.1 ,536/23.2 ,536/23.7
ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 54,000-64,000 daltons and a pI of about 5.6-6.6, and releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and a degree of glucose polymerization of at least 3. The enzyme has a satisfactorily-high thermostability, i.e. it is not substantially inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of trehalose on an industrial scale and in a satisfactorily-high yield.
13 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 426/658,435/100 ,514/54 ,514/61 ,514/777 ,514/778

US-PAT-NO: 6017899
DOCUMENT-IDENTIFIER: US 6017899 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: January 25, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/53,426/658 ,435/100 ,514/54 ,514/61 ,514/777 ,514/778

ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.

14 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/188,435/91.1 ,435/91.5 ,536/25.3

US-PAT-NO: 6013488
DOCUMENT-IDENTIFIER: US 6013488 A
TITLE: Method for reverse transcription
DATE-ISSUED: January 11, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Hayashizaki; Yoshihide	Ibaraki	N/A	N/A	JPX

US-CL-CURRENT: 435/91.51,435/188 ,435/91.1 ,435/91.5 ,536/25.3

ABSTRACT:
A method for preparing a cDNA from a mRNA using a reverse transcriptase wherein reverse transcription is performed at a temperature at which the mRNA does not take a secondary structure, for example, at a temperature of 45.degree. C. or more. The method is performed, for example, using a heat-labile reverse transcriptase in the presence of a substance exhibiting chaperone function having chaperone function such as saccharides. The method is performed, for example, in the presence of metal ions necessary for activation of the reverse transcriptase and a chelating agent for the metal ions such as a deoxynucleotide triphosphate. The method is capable of reverse transcription over the full length of mRNA template even if the mRNA is a long chain mRNA and, as a result, producing a full length cDNA.

8 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

USPT

US-CL-CURRENT: 424/70.13,426/103 ,426/48 ,426/61 ,426/63 ,435/100 ,435/101 ,435/193 ,435/252.3 ,435/320.1 ,435/69.1 ,435/74 ,435/98 ,435/99 ,536/123 ,536/123.1 ,536/123.13 ,536/124 ,536/23.2 ,536/23.7 ,536/4.1

US-PAT-NO: 5993889
DOCUMENT-IDENTIFIER: US 5993889 A
TITLE: Trehalose phosphorylase, its preparation and use
DATE-ISSUED: November 30, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nakada; Tetsuya	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 426/658,424/70.13 ,426/103 ,426/48 ,426/61 ,426/63 ,435/100 ,435/101 ,435/193 ,435/252.3 ,435/320.1 ,435/69.1 ,435/74 ,435/98 ,435/99 ,536/123 ,536/123.1 ,536/123.13 ,536/124 ,536/23.2 ,536/23.7 ,536/4.1

ABSTRACT:
A thermostable trehalose phosphorylase which is obtainable from microorganisms of the genus Thermoanaerobium and which hydrolyzes trehalose in the presence of an inorganic phosphoric acid to form D-glucose and .beta.-D-glucose-1-phosphoric acid. The trehalose phosphorylase can be also prepared by recombinant DNA technology. When the enzyme is allowed to contact with .beta.-D-glucose-1-phosphoric acid as a saccharide donor in the presence of other saccharides, glucosyl-transferred saccharides including glucosyl-D-galactoside, which are conventionally known but scarcely obtainable, can be produced on an industrial-scale and in a relatively-low cost.
15 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 514/23

US-PAT-NO: 5981498
DOCUMENT-IDENTIFIER: US 5981498 A
TITLE: Agent for improving the blood circulation
DATE-ISSUED: November 9, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Fukuda; Shigeharu	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/25,514/23
ABSTRACT:
An agent for improving the blood circulation, which contains as an effective ingredient glycosyl vitamin P such as glycosyl hesperidin and glycosyl rutin. The agent improves the blood circulation in humans to effectively relieve muscular rigidity.
20 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

USPT

US-CL-CURRENT: 435/101,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

US-PAT-NO: 5976856
DOCUMENT-IDENTIFIER: US 5976856 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: November 2, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/201,435/101 ,435/193 ,435/200 ,435/205 ,435/96 ,435/97 ,435/99

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

1 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 435/200,435/252.1 ,435/253.3 ,435/71.1 ,435/877

US-PAT-NO: 5965411
DOCUMENT-IDENTIFIER: US 5965411 A
TITLE: Maltose-trehalose converting enzyme, and preparation and uses thereof
DATE-ISSUED: October 12, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nishimoto; Tomoyuki	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/95,435/200 ,435/252.1 ,435/253.3 ,435/71.1 ,435/877

ABSTRACT:
An enzyme, which has a molecular weight of about 57,000-120,000 daltons on SDS-PAGE and a pI of about 3.8-5.1 on isoelectrophoresis using ampholyte, converts maltose into trehalose and vice versa. The enzyme was isolated from microorganisms of the genera Pimelobacter, Pseudomonas and Thermus. By using the **enzyme, trehalose is readily formed** from a commercially available maltose in an industrial scale and a relatively-low cost. Trehalose and saccharide compositions containing the same, which are preparable with the enzyme, are suitably used in food products, cosmetic compositions and pharmaceutical compositions.

6 Claims, 12 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 12

USPT

US-CL-CURRENT: 435/100,435/101 ,435/72 ,435/74 ,435/97 ,536/123 ,536/123.1 ,536/123.13 ,536/124

US-PAT-NO: 5952204
DOCUMENT-IDENTIFIER: US 5952204 A
TITLE: .beta.-fructofuranosidase, its preparation and uses
DATE-ISSUED: September 14, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nakada; Tetsuya	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/99,435/100 ,435/101 ,435/72 ,435/74 ,435/97 ,536/123 ,536/123.1 ,536/123.13 ,536/124

ABSTRACT:
A .beta.-fructofuranosidase with a molecular weight of 49,000.+-.5,000 daltons on SDS-PAGE, an isoelectric point of 4.6.+-.0.5, an optimum pH of about 5.5-6.0, and an optimum temperature of about 50.degree. C. in the presence of calcium ion. The enzyme acts on saccharides with a .beta.-fructofuranosidic linkage and other substances including other saccharides, sugar alcohols, and alcohols to produce fructosyl-transferred saccharides in a relatively high yield.

11 Claims, 4 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 4

USPT

US-CL-CURRENT: 435/194,435/195 ,435/200 ,435/201 ,435/252.31 ,435/832 ,435/836

US-PAT-NO: 5939308
DOCUMENT-IDENTIFIER: US 5939308 A
TITLE: Heat resistant maltose phosphorylase, process for preparation thereof, bacteria used for preparation thereof, and methods for using the enzyme
DATE-ISSUED: August 17, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Ishii; Keiko	Funabashi	N/A	N/A	JPX
Inoue; Yasushi	Funabashi	N/A	N/A	JPX
Tomita; Tetsuji	Funabashi	N/A	N/A	JPX

US-CL-CURRENT: 435/252.5,435/194 ,435/195 ,435/200 ,435/201 ,435/252.31 ,435/832 ,435/836
ABSTRACT:
This invention relates to heat resistant maltose phosphorylase having an activity of 80% or more of the one untreated after treated in a buffer of pH 6.0, at one temperature of 50 to 60.degree. C. for 15 minutes, a process for preparation thereof, bacteria used for preparation thereof, and processes for preparation of .beta.-glucose-1-phosphoric and trehalose using the enzyme.

By carrying out enzymatic reaction at high reaction temperatures using this enzyme, it is possible to prepare .beta.-glucose-1-phosphoric acid or trehalose industrially advantageously, with lowering of contamination with various germs and shortening of reaction time.
2 Claims, 9 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 435/194,435/195 ,435/200 ,536/123.13

US-PAT-NO: 5935827
DOCUMENT-IDENTIFIER: US 5935827 A
TITLE: Maltose phosphorylase, trehalose phosphorylase, novel strain of genus plesiomonas capable of producing these enzymes and process for producing trehalose
DATE-ISSUED: August 10, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Yoshida; Masahiro	Shizuoka	N/A	N/A	JPX
Nakamura; Nobuyuki	Shizuoka	N/A	N/A	JPX
Horikoshi; Koki	Tokyo	N/A	N/A	JPX

US-CL-CURRENT: 435/100,435/194 ,435/195 ,435/200 ,536/123.13
ABSTRACT:
Disclosed are a novel microorganism (FERM BP-5144) belonging to the genus Plesiomonas and having ability to produce maltose phosphorylase and trehalose phosphorylase required for the enzymatic production of trehalose and novel maltose phosphorylase and trehalose phosphorylase obtainable from the microorganism as well as a process for producing the enzymes. A novel process for enzymatically producing trehalose
(O-.alpha.-D-glucopyranosyl-(1.fwdarw.1)-D-glucopyranoside) is also disclosed.
10 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 424/479,424/59 ,424/70.13 ,426/103 ,426/48 ,426/61 ,426/63 ,435/100 ,435/101 ,435/103 ,435/170 ,435/200 ,435/876 ,435/96 ,435/98 ,514/53 ,514/54 ,536/123 ,536/123.1 ,536/123.13 ,536/127

US-PAT-NO: 5935636
DOCUMENT-IDENTIFIER: US 5935636 A
TITLE: Trehalose and its production and use
DATE-ISSUED: August 10, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nishimoto; Tomoyuki	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 426/658,424/479 ,424/59 ,424/70.13 ,426/103 ,426/48 ,426/61 ,426/63 ,435/100 ,435/101 ,435/103 ,435/170 ,435/200 ,435/876 ,435/96 ,435/98 ,514/53 ,514/54 ,536/123 ,536/123.1 ,536/123.13 ,536/127
ABSTRACT:
Microorganisms which are able to produce maltose/trehalose conversion enzyme, a novel enzyme, are cultivated in nutrient culture media with malose. During the cultivation, the microorganisms readily convert maltose into trehalose to accumulate trehalose in the cultures which yield saccharide mixtures with high trehalose contents when separated from insoluble substances. Removal of contaminant saccharides and subsequent crystallization readily yield trehalose in crystalline trehalose hydrate or anhydrous crystalline form. The trehalose and saccharide mixture containing the same commonly bear desirable properties including mild sweetness and superior stability which render them very useful in a variety of compositions indlucing food products, cosmetics and medicines.
13 Claims, 12 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 12

USPT

US-CL-CURRENT: 435/320.1,435/419 ,435/468 ,536/23.2 ,536/23.7 ,800/284 ,800/317 ,800/317.2

US-PAT-NO: 5925804
DOCUMENT-IDENTIFIER: US 5925804 A
TITLE: Production of trehalose in plants
DATE-ISSUED: July 20, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Hoekema; Andreas	Oegstgeest	N/A	N/A	NLX
Pen; Jan	Leiden	N/A	N/A	NLX
Does; Mirjam Petronella	Amsterdam	N/A	N/A	NLX
Van Den Elzen; Petrus Josephus Maria	Voorhout	N/A	N/A	NLX

US-CL-CURRENT: 800/295,435/320.1 ,435/419 ,435/468 ,536/23.2 ,536/23.7 ,800/284 ,800/317 ,800/317.2

ABSTRACT:
A nucleic acid having (i) a DNA molecule which, when expressed in a plant or plant cell, increases the trehalose content of the plant or plant cell, the DNA molecule encoding an E. coli trehalose phosphate synthase, and (ii) a plant expressible promoter operatively coupled to the DNA molecule. Also, a method for obtaining a plant with increased trehalose production by introducing into a recipient cell of a plant, a plant expressible gene which, when expressed in a plant or plant cell increases the trehalose content of the plant or plant cell. The plant expressible gene is an E. coli trehalose phosphate synthase gene which is operably linked to: a) a transcriptional initiation region that is functional in the plant, and b) a DNA molecule encoding a selectable marker gene that is functional in the plant. The method includes a step of regenerating a plant from the recipient cell under conditions that allow for selection for the presence of the selectable marker gene.

30 Claims, 11 Drawing figures
Exemplary Claim Number: 1,14
Number of Drawing Sheets: 11

USPT

US-CL-CURRENT: 435/100,435/101 ,435/200 ,435/201 ,435/202 ,435/95 ,435/96 ,435/97 ,435/99 ,514/60 ,536/102 ,536/123.1 ,536/124

US-PAT-NO: 5922691
DOCUMENT-IDENTIFIER: US 5922691 A
TITLE: Crystalline maltotetraosyl glucoside, and its production and use
DATE-ISSUED: July 13, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mandai; Takahiko	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/54,435/100 ,435/101 ,435/200 ,435/201 ,435/202 ,435/95 ,435/96 ,435/97 ,435/99 ,514/60 ,536/102 ,536/123.1 ,536/124

ABSTRACT:
Novel crystalline maltotetraosyl glucoside is obtained by crystallizing maltotetraosyl glucoside from a solution of maltotetraosyl glucoside, prepared by either exposing an aqueous solution of maltopentaose to the action of a non-reducing saccharide-**forming enzyme or an aqueous solution which contains trehalose** or a non-reducing saccharide to the action of cyclomaltodextrin glucanotransferase. The crystalline maltotetraosyl glucoside has non-hygroscopicity, non-reducibility, superior solubility, less fermentability, and other satisfactory properties of stabilizing oligopeptides and biologically-substances as well as preventing retrogradation of amylaceous substances. These features render the crystalline maltotetraosyl glucoside very useful in various compositions including foods, beverages, cosmetics, pharmaceuticals and shaped bodies.
19 Claims, 9 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 9

USPT

US-CL-CURRENT: 435/101,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

US-PAT-NO: 5922578
DOCUMENT-IDENTIFIER: US 5922578 A
TITLE: Recombinant thermostable enzyme which forms non-reducing saccharide from reducing amylaceous saccharide
DATE-ISSUED: July 13, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/101 ,435/200 ,435/201 ,435/202 ,435/205 ,435/96 ,435/99

ABSTRACT:
Disclosed is a recombinant thermostable enzyme which has a molecular weight of about 69,000-79,000 daltons and a pI of about 5.4-6.4, and forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of glucose polymerization of at least 3. The enzyme has satisfactorily high thermostability, i.e. it is substantially not inactivated even when incubated in an aqueous solution (pH 7.0) at 85.degree. C. for 60 min, and this facilitates the production of such non-reducing saccharides on an industrial scale and in a satisfactorily-high yield.

7 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

USPT

US-CL-CURRENT: 426/658,435/101 ,435/252.1 ,435/253.1 ,435/99 ,514/53 ,514/54 ,514/60 ,536/123.1 ,536/123.12 ,536/123.13 ,536/126

US-PAT-NO: 5922580
DOCUMENT-IDENTIFIER: US 5922580 A
TITLE: Non-reducing saccharide-forming enzyme, its preparation and uses
DATE-ISSUED: July 13, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Maruta; Kazuhiko	Okayama	N/A	N/A	JPX
Kubota; Michio	Osaka	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/100,426/658 ,435/101 ,435/252.1 ,435/253.1 ,435/99 ,514/53 ,514/54 ,514/60 ,536/123.1 ,536/123.12 ,536/123.13 ,536/126
ABSTRACT:
Disclosed are novel non-reducing saccharide-forming enzyme, and its preparation and uses. The enzyme is obtainable from the culture of microorganisms such as Rhizobium sp. M-11 (FERM BP 4130) and Arthrobacter sp. Q36 (FERM BP-4316), and capable of forming non-reducing saccharides having a trehalose structure when allowed to act on reducing partial starch hydrolysates. Glucoamylase and .alpha.-glucosidase readily yield trehalose when allowed to act on the non-reducing saccharides. These non-reducing saccharides and trehalose are extensively useful in food products, cosmetics and pharmaceuticals.
13 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/100,435/101 ,435/72 ,435/74 ,435/95 ,435/96 ,435/98 ,435/99 ,536/123.1 ,536/123.13

US-PAT-NO: 5919668
DOCUMENT-IDENTIFIER: US 5919668 A
TITLE: Non-reducing saccharide and its production and use
DATE-ISSUED: July 6, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mandai; Takahiko	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/97,435/100 ,435/101 ,435/72 ,435/74 ,435/95 ,435/96 ,435/98 ,435/99 ,536/123.1 ,536/123.13

ABSTRACT:
In the production of non-reducing saccharides such as trehalose, alpha-glycosyl trehaloses and alpha-glycosyl alpha-glycosides where a solution of liquefied starch is subjected either to non-reducing saccharide-forming **enzyme or non-reducing saccharide-forming enzyme and trehalose**-releasing enzyme, combinations with starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase improve the yields for such non-reducing saccharides to levels which are hardly attainable only with reducing-saccharide**-forming enzyme and trehalose**-releasing enzyme. The non-reducing saccharides and less reducing reducing saccharides containing the same commonly bear a variety of desirable properties which make them useful in a variety of compositions including food products, cosmetics and medicines.
22 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17

USPT

US-CL-CURRENT: 127/58,127/60 ,127/61

US-PAT-NO: 5916371
DOCUMENT-IDENTIFIER: US 5916371 A
TITLE: Crystalline powdery saccsharide, its preparation and uses
DATE-ISSUED: June 29, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Mukai; Kazuhisa	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 127/29,127/58 ,127/60 ,127/61

ABSTRACT:
A stable crystalline powdery saccharide having a crystallinity of 40% or more, less hygroscopicity, satisfactory fluidity, and beneficial handleability, which is obtainable from an aqueous solution, containing trehalose and a different saccharide(s) crystallizable in the presence of trehalose, by crystallizing the trehalose along with the different saccharide(s).

14 Claims, 6 Drawing figures
Exemplary Claim Number: 5
Number of Drawing Sheets: 3

USPT

US-CL-CURRENT: 426/658,435/100 ,435/101 ,435/99 ,514/54 ,514/61 ,536/123.1 ,536/123.13

US-PAT-NO: 5916881
DOCUMENT-IDENTIFIER: US 5916881 A
TITLE: High trehalose content syrup
DATE-ISSUED: June 29, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Okada; Katsuhide	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 514/53,426/658 ,435/100 ,435/101 ,435/99 ,514/54 ,514/61 ,536/123.1 ,536/123.13

ABSTRACT:
A non- or substantially non-crystalline high trehalose content syrup, which dissolves trehalose in an amount over the water solubility and dissolves other oligosaccharide having a trehalose structure within the molecule. The oligosaccharide acts as a trehalose crystallization inhibitory agent and makes the syrup stable, free of or substantially free of crystallization at ambient temperature, and free from bacterial contamination. Examples of the oligosaccharide are monoglucosyltrehalose, diglucosyltrehalose, and triglucosyltrehalose.

21 Claims, 0 Drawing figures
Exemplary Claim Number: 1

USPT

US-CL-CURRENT: 435/41,536/123.13 ,549/263 ,549/295 ,549/297

US-PAT-NO: 5912361
DOCUMENT-IDENTIFIER: US 5912361 A
TITLE: Process for producing D-glucuronolactone
DATE-ISSUED: June 15, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tsuchioka; Toshiki	Hiroshima-ken	N/A	N/A	JPX
Yamaguchi; Tadashi	Hiroshima-ken	N/A	N/A	JPX
Yuuen; Kunihiko	Okayama-ken	N/A	N/A	JPX
Chaen; Hiroto	Okayama-ken	N/A	N/A	JPX

US-CL-CURRENT: 549/311,435/41 ,536/123.13 ,549/263 ,549/295 ,549/297

ABSTRACT:
Trehalose is oxidized to give oxidized trehalose which then is hydrolyzed to produce D-glucuronolactone which is thereafter recovered to realize high-yield and low-cost production of D-glucuronolactone.

18 Claims, 0 Drawing figures
Exemplary Claim Number: 1

USPT

US-CL-CURRENT: 435/101,435/72 ,435/74 ,435/96 ,435/97 ,435/98 ,435/99 ,536/123.1 ,536/123.13 ,536/127

US-PAT-NO: 5912330
DOCUMENT-IDENTIFIER: US 5912330 A
TITLE: Crystalline maltosyl glucoside, and its production and use
DATE-ISSUED: June 15, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Tabuchi; Akihiko	Okayama	N/A	N/A	JPX
Shibuya; Takashi	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 536/4.1,435/101 ,435/72 ,435/74 ,435/96 ,435/97 ,435/98 ,435/99 ,536/123.1 ,536/123.13 ,536/127

ABSTRACT:
Novel crystalline maltosyl glucoside is obtained by crystallizing maltosyl glucoside from a maltosyl glucoside solution, prepared by exposing either an aqueous solution containing trehalose and an .alpha.-glucosyl saccharide or an aqueous solution containing a reducing partial starch hydrolysate to the action of a saccharide-transferring enzyme. The crystalline maltosyl glucoside has non-hygroscopicity, non-reducibility, superior solubility, less fermentability, and other properties of stabilizing oligopeptides and biologically-substances as well as preventing retrogradation of amylaceous substances. These features render it very useful in various compositions including foods, beverages, cosmetics, pharmaceuticals and shaped bodies.

20 Claims, 8 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

USPT

US-CL-CURRENT: 435/252.3,435/320.1 ,530/350 ,536/23.2 ,536/23.7

US-PAT-NO: 5910436
DOCUMENT-IDENTIFIER: US 5910436 A
TITLE: Trehalose phosphorylase, its preparation and uses
DATE-ISSUED: June 8, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Nakada; Tetsuya	Okayama	N/A	N/A	JPX
Kubota; Michio	Okayama	N/A	N/A	JPX
Chaen; Hiroto	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/193,435/252.3 ,435/320.1 ,530/350 ,536/23.2 ,536/23.7

ABSTRACT:
A thermostable trehalose phosphorylase which is obtainable from microorganisms of the genus Thermoanaerobium and which hydrolyzes trehalose in the presence of an inorganic phosphoric acid to form D-glucose and .beta.-D-glucose-1-phosphoric acid. The trehalose phosphorylase can be also prepared by recombinant DNA technology. When the enzyme is allowed to contact with .beta.-D-glucose-1-phosphoric acid as a saccharide donor in the presence of other saccharides, glucosyl-transferred saccharides including glucosyl-D-galactoside, which are conventionally known but scarcely obtainable, can be produced on an industrial-scale and in a relatively-low cost.

6 Claims, 5 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

USPT

US-CL-CURRENT: 435/101,435/72 ,435/95 ,435/96 ,435/97 ,435/99 ,514/53 ,536/123.1 ,536/123.13

US-PAT-NO: 5908767
DOCUMENT-IDENTIFIER: US 5908767 A
TITLE: Non-reducing saccharide and its production and use
DATE-ISSUED: June 1, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kubota; Michio	Okayama	N/A	N/A	JPX
Sugimoto; Toshiyuki	Okayama	N/A	N/A	JPX
Miyake; Toshio	Okayama	N/A	N/A	JPX

US-CL-CURRENT: 435/100,435/101 ,435/72 ,435/95 ,435/96 ,435/97 ,435/99 ,514/53 ,536/123.1 ,536/123.13

ABSTRACT:
Non-reducing saccharides including alpha-glycosyl trehaloses, alpha-glycosyl alpha-glycosides and trehalose are easily produced by cultivating microorganisms capable of producing non-reducing saccharide-forming enzyme in nutrient culture media which contain reducing partial starch hydrolysates with glucose polymerization degrees of 3 or higher. The yields for these saccharides are significantly improved by further subjecting the reducing partial starch hydrolysates to starch-debranching enzyme and/or cyclomaltodextrin glucanotransferase in or not in culture media. The resultant non- or less-reducing saccharides commonly bear desirable properties in addition to a mild and gentle sweetness. Thus they would find extensive uses in a variety of compositions including food products, cosmetics and medicines.
17 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 17